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Research article

Perinatal protein malnutrition alters expression of miRNA biogenesis genes Xpo5 and Ago2 in mice brain

Bruno G. Berardino, Estefanía A. Fesser, Eduardo T. Cánepa*

Laboratorio de Neuroepigenética, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas, Ciudad de Buenos Aires, Argentina

HIGHLIGHTS

- Perinatal protein malnutrition delays physical and neurological development.
- Maternal protein deficiency reduces hippocampal weight.
- Xpo5 and Ago2 mRNA levels are altered in malnourished mice.
- Induced expression of Xpo5 correlates with high levels of overall mature miRNAs.

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ABSTRACT

Due to its widespread incidence, maternal malnutrition remains one of the major non-genetic factors affecting the development of newborn's brain. While all nutrients have certain influence on brain maturation, proteins appear to be the most critical for the development of neurological functions. An increasing number of studies point out that the effects of early-life nutritional inadequacy has long lasting effects on the brain and lead to permanent deficits in learning and behavior. Epigenetic mechanisms provide a potential link between the nutrition status during critical periods and changes in gene expression that may lead to disease phenotypes. Among those epigenetic mechanisms microRNAs (miRNAs) emerge as promising molecules for the link between nutrition and gene expression due to their relevance in many central nervous system functions. The objective of the current study was to evaluate the impact of perinatal protein malnutrition on the development of male and female mice offspring and to analyze the expression of the genes involved in the miRNA biogenesis pathway in different mouse brain structures. We demonstrated that early nutritional stress such as exposition to a protein-deficient diet during gestation and lactation reduced the hippocampal weight, delayed offspring's development and deregulated the expression of Xpo5 and Ago2 genes in hippocampus and hypothalamus of weanling mice. Moreover, an overall increase in mature miRNAs was consistent with the induction of Xpo5 mRNA. Altered miRNA biogenesis could modify the availability and functionality of miRNA becoming a causal factor of the adverse effects of protein malnutrition.

1. Introduction

Nutrition plays a crucial role in the maturation and functional development of the central nervous system. Inadequate nutrition before birth and during the early postnatal life can seriously interfere with brain development and lead to behavioral and neurological disorders such as learning disabilities and psychiatric diseases [1,2]. In particular, protein malnutrition causes alterations in the developmental time course of dentate gyrus, in the morphology of hippocampal cells and in the number and distribution of neurotransmitter receptors [3,4]. In addition, protein deficiency induces changes in fetal programming of neuropeptide expression in particular those serving as activating signals to the hypothalamic-pituitary-adrenal (HPA) axis [5].

Increasing epidemiological evidence suggests that maternal nutrition and environmental conditions early in development play an important role in susceptibility to disease in later life [6,7]. Epigenetic mechanisms provide a potential link between the nutrition status during critical periods and changes in gene expression that may lead to disease phenotypes. A growing body of evidence from experimental animal studies supports the role of epigenetics

* Corresponding author at: Laboratorio de Neuroepigenética, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II piso 4, C1428EGA Ciudad de Buenos Aires, Argentina.
E-mail address: ecanepa@qb.fcen.uba.ar (E.T. Cánepa).

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in disease susceptibility during developmental periods, including perinatal and early postnatal period [8,9].

In the past few years, several evidences demonstrate that microRNAs (miRNAs) are key players in epigenetic mechanisms. They constitute a class of small non-protein coding RNA molecules, around 22 nucleotides in length, which affect the activity of mRNAs, directly degrading it and/or preventing its translation [10,11]. Regulation by miRNAs appears to be the most abundant mode of post-transcriptional regulation. This is because hundreds of miRNA genes, each regulating a diverse set of downstream targets, take part in practically all cellular processes, whether in health or disease [8,12]. miRNAs are abundantly expressed in the nervous system and a relation between miRNAs and the regulation of neuronal synaptic and structural plasticity has been demonstrated in different model systems. More recently, a role of miRNAs influencing learning and memory capacities probably by regulating dendrite morphogenesis during early development has been reported [13,14].

The components of the miRNA biogenesis and processing machinery are well conserved across the animal kingdom. miRNA transcription occurs via RNA polymerase II or RNA polymerase III, upstream of intergenic miRNAs or miRNAs residing in introns of coding or noncoding genes generating primary transcripts known as pri-miRNA. After transcription, pri-miRNA is processed by RNase III domain-containing protein Drosha in association with the RNA binding protein encoded by DiGeorge syndrome critical region gene 8 (Dgcr8)/Pasha. The result of this processing is a precursor-miRNA (pre-miRNA) which is then exported by the nuclear transport factor exportin 5 (Xpo5) to the cytoplasm. Once there the RNase Dicer further processes the pre-miRNA into a 21–22 nt duplex, and one of both strands is then loaded into the Argonaute 2 (Ago2)-protein-containing complex called miRNA-induced silencing complex (miRISC). Within miRISC, the single-stranded mature miRNA forms partial complementary contacts on target mRNAs, which typically mediate mRNA degradation or translational inhibition [15,16].

Deregulation of miRNA biogenesis pathway is an emerging mechanism involved in several neurodegenerative disorders. Recently, it was demonstrated that loss of Dicer is sufficient to cause progressive degeneration of spinal motor neurons and that global downregulation of miRNAs is a common molecular denominator of multiple forms of human amyotrophic lateral sclerosis. In addition, diminished level of Dicer1 expression was associated with post-traumatic stress disorder [17–19]. Moreover, downregulation and/or mislocalization of Drosha and Dgcr8 were potentially involved in frontotemporal dementia and in Fragile-X associated ataxia [20,21].

These evidences highlight the essentiality of a suitable regulation of miRNA biogenesis machinery to maintain brain integrity. However, no studies concerning the effects of malnutrition on this mechanism have been reported. In this regard, the objective of the current study was to evaluate the effects of perinatal protein malnutrition on the expression of the components of miRNA biogenesis pathway in different mice brain structures. Sex is an important factor to consider regarding the effects of early-life stress. In particular, maternal undernutrition has been described to be sex-dependent in experimental models. It was observed that only female offspring from dams subjected to protein restriction during pregnancy exhibited traits of hopelessness behavior [22]. In addition, low-protein diet during rat pregnancy induced insulin resistance and hypertension only in male offspring in adulthood [23,24]. Taking into account these evidences we extended our study to both sexes. Our results show that perinatal protein restricted offspring display altered expression of Xpo5 and Ago2 genes. Moreover, we observed an overall increase in mature miRNAs that was consistent with Xpo5 induction. Our results suggest that availability and functionality of miRNAs could be impaired under early life nutritional deficiency.

2. Materials and methods

2.1. Animals and diets

CF-1 mice from the colony of the *Bioterio Central*, *Facultad de Ciencias Exactas y Naturales* (University of Buenos Aires), were used for all experiments. The diets employed in this study were prepared according to the AIN-93 Final Report [25] and purchased from Research Diets Inc. (New Brunswick, NJ). Nutrient composition of normal-protein (NP) and low-protein (LP) diets are available upon request. NP and LP diets contain 20% and 8% of casein as the sole source of proteins, respectively.

Experiments were performed in accordance with local regulations and the National Institutes of Health (NIH) Guide of the Care and Use of Laboratory Animals (NIH publication 80-23/96) and were previously approved by the Ethical Committee (CICUAL – Protocol N° 0024) of the *Facultad de Ciencias Exactas y Naturales* (University of Buenos Aires). Experimental design is described in Supplementary Material.

2.2. Tissue RNA isolation and reverse transcription

Pups were weaned at PD21 and killed by cervical dislocation. After tissue isolation brain was dissected to obtain the hypothalamus, the hippocampus, the olfactory bulb and the whole cortex, and then snap frozen in liquid nitrogen until use. Each brain section was homogenized in RNAZol (Molecular Research Center), which allows an RNA separation based on molecular weight. Small (<200 nt) and large (>200 nt) RNA fractions was extracted following manufacturer's instructions. One μg or 250 ng aliquots of large RNA or small RNA fractions respectively was reverse transcribed (RT) to cDNA using MMLV (Promega). For the large RNA fraction 2 μl of 10 μM Oligo-dT (Genbiotech, Argentina) was used. For the small RNA fraction a miRNA-specific primer consisting of a stem loop oligonucleotide was used for each miRNA and a specific reverse primer was used for U6 (Supplementary Table 1). For 18S ribosomal RNA retrotranscription random primers were used instead of oligo-dT. A non-transcribed control (NRT) including RNA and all the other reagents but no retrotranscriptase was included.

2.3. Real time-PCR analysis

Primers for quantitative real-time PCR (qPCR) were designed using Geneious software and purchased from Genbiotech, Argentina. Primer sequences are listed in Supplementary Table 2. RT-qPCR was performed with 5 μl of RT product diluted 1:10, 10 μl of primers forward and reverse mix (0.5 μM each), 0.2 μl of Taq Polymerase (Invitrogen, Brazil), 2.5 μl of 10X Enzyme Buffer, 2 μl of 50 mM $MgCl_2$, 2.5 μl of 2 mM dNTPs and adjusted to a total volume of 25 μl per reaction with nuclease free water. qPCR amplification and detection was performed in 96-well plates using Stratagene Mx3005P machine. The reactions were initialized at 95 °C for 10 min, denatured at 95 °C for 15 s, annealed at 63 °C for 20 s and elongated at 72 °C for 25 s for 40 cycles. The dissociation curve was achieved by melting the DNA at 95 °C for 1 min, incubating the DNA at 55 °C for 30 s and followed by a ramp up to 95 °C for 30 s. Primers were optimized using a standard curve of pooled cDNA. A non-template control (nuclease free water) and a non-transcribed control (NRT) were included on each plate to check for any contamination. Samples and controls were analyzed in triplicate. The level of mRNA expression was normalized to the geometric mean of the 3 more consistently expressed housekeeping genes among the following: Gapdh, Pgk1, Hprt1, Hspcb and 18S.

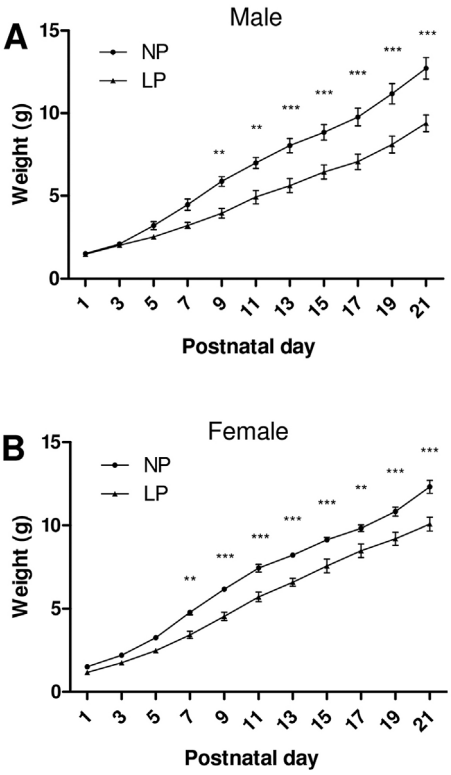

Fig. 1. Weight gain curves of NP and LP mice over the lactation period. A, Body weight of male offspring. B, Body weight of female offspring. Values are means ± SEM (n = 8–10). ** p < 0.01; *** p < 0.001.

The small nuclear RNA U6 was used to relativize miRNA expression. Differential expression was assessed by comparing the initial template quantity (N0) by a linear regression using LinReg PCR. All values were expressed relative to NP treatment.

2.4. Statistical analysis

Statistical tests used throughout the paper are described in Supplementary Material. When data complied with normal distribution and equal variances between treatments, Student's *t*-test was applied. Welch's correction was used when data did not meet homoscedasticity criteria. Mann Whitney non-parametric test was employed when data failed to pass normality testing. For parameters analyzed in the same individual during a series of days (weight, food intake, surface righting reflex), a two-way ANOVA with repeated measures and Bonferroni post-test was used to compare between treatments. In order to analyze if there were significant differences in the proportion of mice that developed a landmark per day a Chi-square test was used. Data were tested with Grubbs' method in order to detect the presence of outliers [26]. This is a statistical test used to detect outliers in a univariate dataset assumed to come from a normally distributed population. Values that were considered outliers ($\alpha = 0.05$) were removed from the dataset before statistical analysis.

3. Results

3.1. Protein malnutrition reduces hippocampal weight

The body weight of both male and female pups was measured during lactation. No differences were observed at birth between offspring from malnourished dams (LP) compared with offspring from normally fed dams (NP) neither in female nor in male mice (Fig. 1).

Table 1
Hippocampus and brain weight.

	Female		Male	
	NP	LP	NP	LP
Brain				
WM (mg)	382.40	368.90	397.80	371.00*
SD	28.96	28.96	30.91	25.96
Hippocampus				
WM (mg)	16.31	13.68*	18.25	15.02##
SD	3.16	3.36	3.79	2.18
Hippocampus/Brain				
Percentage (%)	4.27	3.70†	4.56	4.04†
SD	0.80	0.84	0.79	0.47

NP, normal protein diet; LP, low protein diet.
WM, Weight Mean.
SD, Standard Deviation.
* Significantly different (p < 0.05) from NP; Unpaired *t*-test.
† Significantly different (p < 0.05) from NP; Mann Whitney test.
Significantly different (p < 0.01) from NP; Welch's correction.

However, offspring of low protein dams had slower early postnatal growth, resulting in lower body weight of female pups from postnatal day (PD) 7 (p < 0.01) and male pups from PD9 (p < 0.01).

We next evaluated the impact of dam's malnutrition during pregnancy and lactation on the physical and neurological development of pups. LP mice showed a delay in physical and neurological parameters, which were consistent with previous reports by Bellusscio et al. (Supplementary Fig. 1) [22]. These observations validated the maternal malnutrition paradigm used in this work.

To further assess the effect of perinatal malnutrition we determined the weight of the whole brain and hippocampus in mice from both groups since it has been reported that development of this region is affected by environmental stress [27]. Notably, we observed that hippocampus was affected by the nutritional condition (Table 1). Both male and female LP offspring exhibited a reduced hippocampus-to-brain ratio when compared to NP offspring (p < 0.05). As a whole these results show that physical growth and neurological development are impaired by maternal protein malnutrition during gestation and lactation.

3.2. Body weight and food intake of dams during pregnancy and lactation

The body weight of dams was measured throughout gestation and lactation. Pregnant dams of both groups exhibited a similar increase in body weight during the gestation period (Supplementary Fig. 2). Nevertheless, after given birth, the weight of LP mothers presented a tendency to be lower than that of NP dams, showing a significant difference at lactation day 4 (p < 0.01) and 16 (p < 0.05) (Fig. 2A).

Offspring's nutritional condition depends almost exclusively on mother's nutrients and caloric intake. Therefore, we measured the food intake of dams from both groups during pregnancy and lactation. Equal amount of food was consumed by NP and LP dams throughout pregnancy (Supplementary Fig. 3). After birth, we observed that NP dams consumed more food per day than their LP counterparts from lactation day 15 until weaning (p < 0.05 and p < 0.001) (Fig. 2B). However, the relative food intake, i.e. total quantity of food per day relative to the number of lactating pups, was similar for both groups (Fig. 2C). Since both diets were isocaloric, these results strongly suggest that the above described physical and neurological differences between offspring from NP and LP groups should be due to the protein deficiency and not to a lesser quantity of food and/or calories consumed by their mothers.

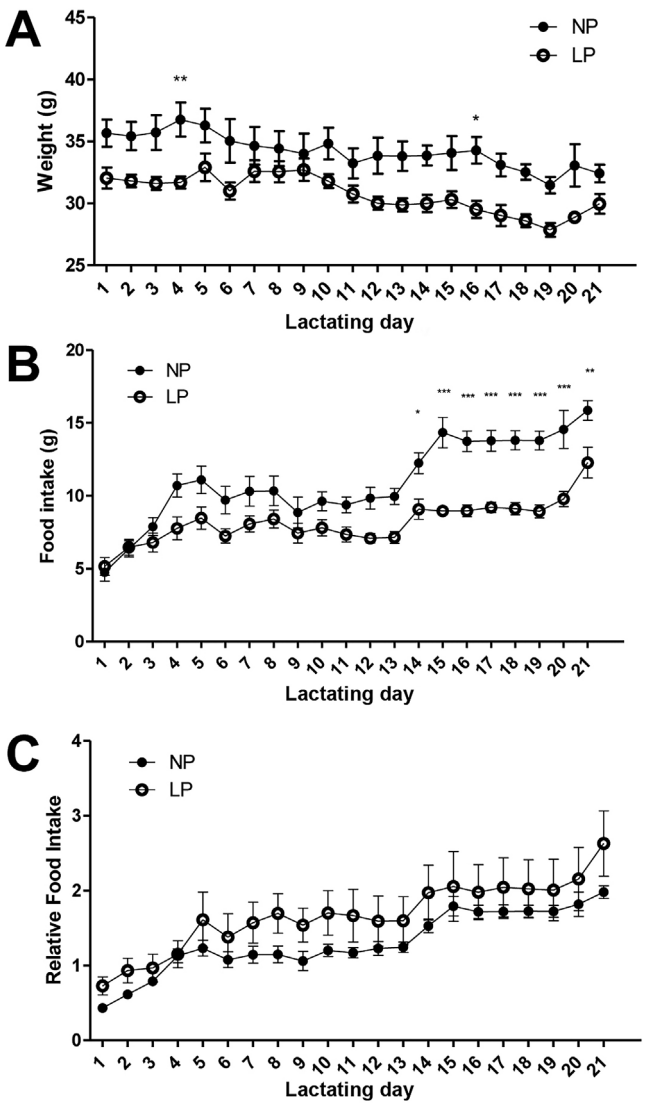

Fig. 2. Weight gain curves and food intake of NP and LP dams over the lactation period. A, Body weight of dams during lactation. B, Food intake of dams during lactation. C, Food intake of dams during lactation relative to number of pups in the litter. Values are means ± SEM (n = 6–7). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3. Early protein malnutrition disturbs miRNA biogenesis pathway

Small non-coding miRNAs are known to play an important function in both brain development and its adaptation to stress. Therefore, the effect of early protein malnutrition on the expression of genes involved in miRNA biogenesis was determined. Hippocampus and hypothalamus were used in this experiment due to their role in stress response. Hippocampus and olfactory bulb were also selected because they are capable to produce neurogenesis and to respond to changes in environment through synaptic plasticity. Lastly, the cerebral cortex has been shown to have a particular susceptibility to oxidative stress [28,29], which may be caused by a protein deficient diet.

We observed that levels of Xpo5 mRNA were increased in both hippocampus and hypothalamus regions of male LP mice with respect to the levels determined in male NP mice ($p < 0.05$) (Fig. 3A and C). A similar increase, but only in hippocampus, was observed in female LP offspring ($p < 0.05$) (Fig. 3B). On the other hand, mRNA levels of Ago2, the catalytic component of RISC, were diminished in the same brain structures of male LP mice ($p < 0.05$) (Fig. 3A and C). Remarkably, female LP offspring exhibited an increased expression of Ago2 in hypothalamus unlike what it was observed in male offspring ($p < 0.01$) (Fig. 3D).

Hippocampus and hypothalamus expression of Drosha and Dgcr8, members of the called microprocessor complex, and the cytoplasmic RNAse III Dicer were not modified by perinatal protein malnutrition neither in male nor in female offspring (Fig. 3). Finally, expression of none of the components of the miRNA biogenesis pathway analyzed was modified in cortex and olfactory bulb (Supplementary Fig. 4).

3.4. High levels of mature miRNAs correlate with induced Xpo5 mRNA

We next attempted to address whether the upregulation of Xpo5 mRNA in LP mice is functionally relevant. We rationalize that an overall increase in mature miRNA levels will be observed in those brain regions where Xpo5 expression is induced. To test this, the expression of ten miRNAs randomly selected from those expressed in the brain was determined in the hippocampus of both NP and LP mice. We observed that the levels of 70% and 50% of the miRNAs, in male and female respectively, were increased in the hippocampus of LP mice when compared with their NP counterparts (Fig. 4). Six out of the ten miRNAs analyzed in hippocampus were measured also in hypothalamus. In this case, 33% of them showed significantly higher expression in male LP mice. Considering the induction of Xpo5 expression in the hippocampus (56%) and in the hypothalamus (28%) of male LP mice (Fig. 3), we can observe that the greater the mRNA levels of Xpo5 the greater the number of miRNAs whose levels are increased (Fig. 4). Remarkably, female hypothalamus displayed no modification in miRNA levels in both NP and LP mice coincidently with the absence of change in Xpo5 expression in this region. It is noteworthy that none of the miRNAs exhibited an opposite behavior, i.e. lower levels in the LP group than in the NP group.

These results strongly support that a close correlation exists betweenXpo5 gene expression and overall levels of mature miRNA.

4. Discussion

In the current work, we studied the effect of protein malnutrition during gestation and lactation on miRNA biogenesis pathway in male and female weanling mice. A consequence of a protein restriction for the offspring of both sexes is a sustained deficit in body weight. By PD7 and PD9 female and male LP mice, respectively, began to gain less weight per day than their NP counterparts. Moreover, in accordance with previous reports we observed a delayed physical and neurological development [22]. The present findings suggest that perinatal exposure to protein malnutrition affects embryological mechanisms responsible for the correct individual development [22,30].

We demonstrated that the expression of Xpo5 and Ago2 genes are altered in hippocampus and hypothalamus of 3-weeks-old mice exposed to protein malnutrition during perinatal period. Furthermore, LP mice showed a reduction of hippocampus size and a lower hippocampus-to-brain ratio. Smaller hippocampal volumes have been reported in different clinical populations of patients suffering from psychiatric disorders [31]. Those variations in hippocampal volume are considered as a consequence of the neurotoxic effects of stress leading to increased vulnerability for cognitive and emotional impairments [32,33].

The observed reduction in hippocampus size may result from several causes including augmented cell death and/or diminished neurogenesis. In this regard, it was reported that malnutrition during rat gestation and lactation results in an increase in the level of

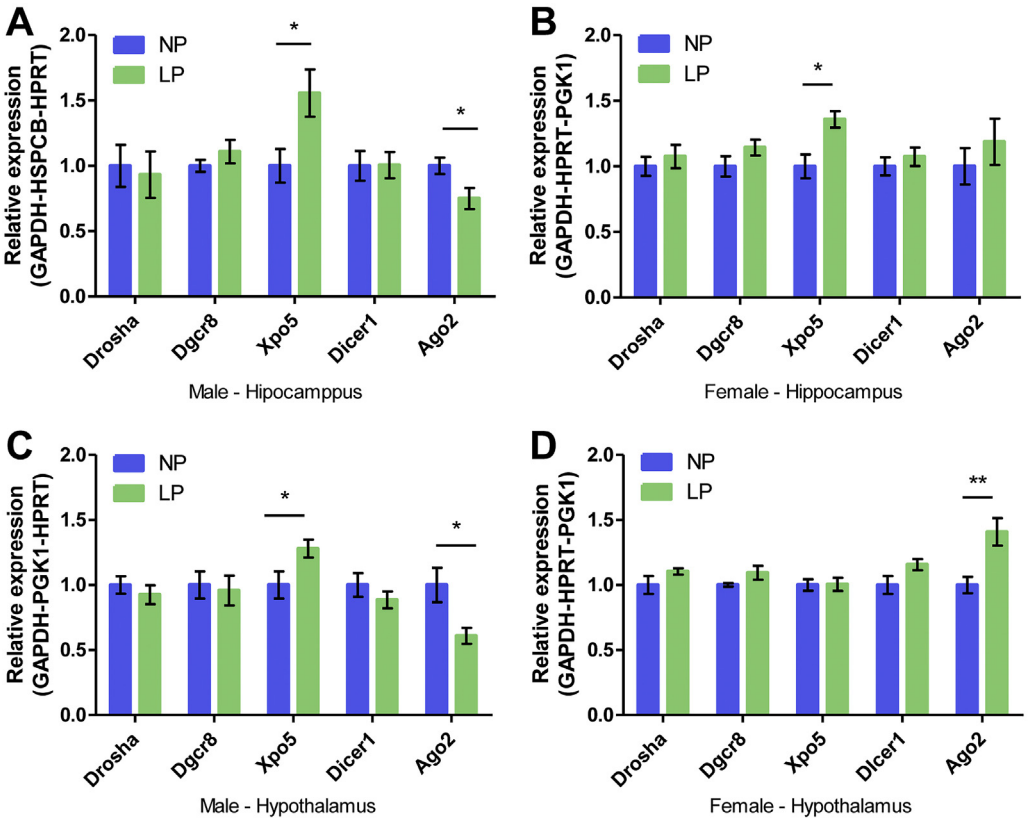

Fig. 3. miRNA biogenesis genes expression in hippocampus and hypothalamus of NP and LP male and female offspring at PD21. A, Gene expression in male hippocampus. B, Gene expression in female hippocampus. C, Gene expression in male hypothalamus. D, Gene expression in female hypothalamus. Values are means ± SEM (n = 6–10). * $p < 0.05$; ** $p < 0.01$.

apoptotic cells in the dentate gyrus of the hippocampal formation [34]. According to this work, malnutrition may induce cell death related to the availability of neurotrophic factors. On the other hand, early life adversities like stress, inadequate nutrition and infection has been reported to result in lifelong alterations in hippocampal related cognitive functions, at least partly due to changes in hippocampal neurogenesis [35]. Both neuronal cell death pathways and the entire process of neurogenesis are modulated by miRNAs [36,37]. Thereby, deregulated miRNA biogenesis pathway challenged by nutritional stress may affect the availability of cytoplasmic miRNA leading to disruption of both processes.

Optimal function of the hippocampal formation is critical for modulation of the hypothalamic-pituitary axis and regulation of the stress response, deregulation of which is observed in almost half of individuals with emotional dysfunction [38,39]. In this regard, our results showing altered levels of Xpo5 and Ago2 mRNAs in hypothalamus of LP mice could be related to these observations. Notably, changes in the expression of miRNA biogenesis components in female LP mice were somewhat different than it was observed in males. Upregulation of Xpo5 was also observed in hippocampus. Conversely, Ago2 mRNA levels were increased in hypothalamus of LP female mice. While the origin of these differences between male and female is difficult to explain, its consequences may be related to the different gender susceptibility to emotional dysfunction in response to early life stress [40,41].

The data presented in this study allow us to sustain a functional relevance of the induced mRNA levels of Xpo5 in hippocampus and hypothalamus of protein malnourished mice. Two experimental evidences support this statement. First, there was a direct correlation between the extent of Xpo5 mRNA induction and the number of mature miRNAs whose levels were increased in hippocampus and hypothalamus of LP mice. Second, there were no changes in miRNA levels when Xpo5 was not induced as observed in the female hypothalamus suggesting a causal relationship between Xpo5 induction and the increased levels of mature miRNAs. Together, these findings underscore the biological significance of Xpo5 mRNA deregulation triggered by perinatal protein malnutrition which leads to an aberrant increase in mature miRNA levels.

As an essential component of the miRISC, Ago2 is able to control mRNA expression using miRNAs as guide molecules to interact with specific mRNAs and thereby induce mRNA decay and translational suppression. Thus, altered mRNA levels of Ago2 observed in hippocampus and hypothalamus of LP mice suggests that the expression of certain mRNAs would be substantially affected. Taken together, our results indicate that the availability of miRNAs in the cytoplasm, due to the induction of Xpo5 mRNA, and its functionality, due to altered levels of Ago2 mRNA, are strongly compromised in mice that were subjected to protein malnutrition during the pre- and postnatal periods.

miRNAs are able to modulate the generation of reactive oxygen species (ROS) and the expression of antioxidant defense genes. Meanwhile, redox stress can alter the miRNA biogenesis and processing pathway [42]. In this regard, dietary protein is important for antioxidant mechanisms and it has been demonstrated that protein malnutrition may lead to an increase in oxidative damage by diminishing antioxidant defenses of the tissue [43]. Thus, protein deficiency and deregulation of miRNA biogenesis would result in augmented ROS formation and increased vulnerability to brain injury. Remarkably, two miRNAs that we have determined to be induced in hippocampus of LP offspring, miR-124 and miR-26a, were reported to target mRNAs that codified for protein members

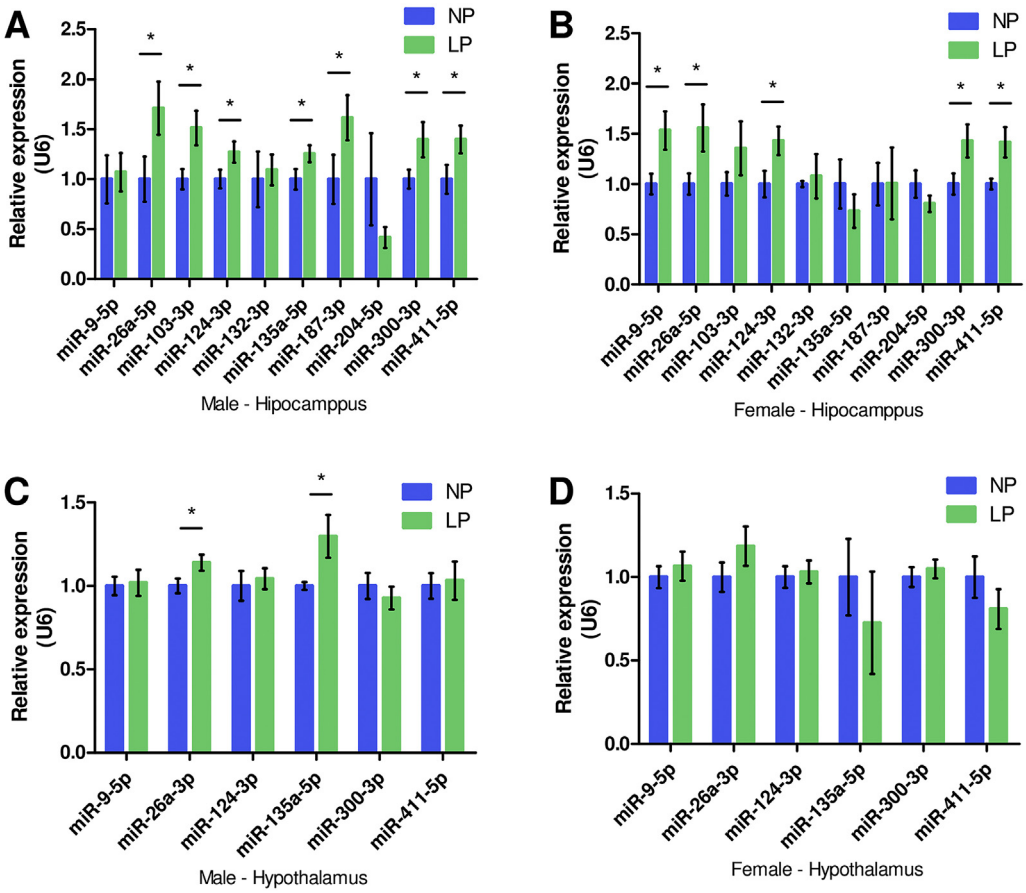

Fig. 4. Expression of miRNAs in hippocampus and hypothalamus of NP and LP male and female offspring at PD21. A, miRNA expression in male hippocampus. B, miRNA expression in female hippocampus. C, miRNA expression in male hypothalamus. D, miRNA expression in female hypothalamus. Values are means ± SEM (n = 6–10). * $p < 0.05$.

of Gst and Txn families, respectively, enzymes that belong to the antioxidant system operating in brain [44,45].

Interestingly, abnormal miRNA biogenesis pathway, specifically changes in Xpo5 expression, has been related with other diseases. Transgenic mice model of Huntington's disease, a genetic neurodegenerative disease caused by abnormal expansion of CAG repeats in the gene encoding huntingtin, exhibit an increased expression of Xpo5, Drosha and Dgcr8 at five months old [46]. In addition, evidences that inactivating mutations of Xpo5, lacking a C-terminal region, occur in human tumors with microsatellite instability was provided, supporting a role of Xpo5 as a tumor-suppressor gene [47]. Similarly, altered levels of Ago2 have been found

in neurological disorders such as epilepsy [48] and cocaine addiction [49]. Moreover, Ago2 has also been found overexpressed in carcinomas and has been proved to be related to aspects of cancers like tumor cell growth and cell survival [50].

In summary, we demonstrated for the first time to our knowledge that early nutritional stress such as exposition to a deficient protein diet during gestation and lactation deregulates the miRNA biogenesis pathway in hippocampus and hypothalamus of weanling mice. Altered miRNA biogenesis modifies the availability and thus could affect the functionality of miRNAs becoming a causal factor of the negative effects of protein malnutrition.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2017.03.012.

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