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A moderate diet restriction during pregnancy alters the levels of endocannabinoids and endocannabinoid-related lipids in the hypothalamus, hippocampus and olfactory bulb of rat offspring in a sex-specific manner

María Teresa Ramírez-López[1], Mariam Vázquez[1,2], Ermelinda Lomazzo[3], Clementine Hofmann[3], Rosario Noemi Blanco[1], Francisco Alén[1], María Antón[1], Juan Decara[2], Rocío Arco[2], Laura Orio[1], Juan Suárez[2,4], Beat Lutz[3], Raquel Gómez de Heras[1]*, Laura Bindila[3‡], Fernando Rodríguez de Fonseca[1,2‡]*

1 Departamento de Psicobiología. Facultad de Psicología, Universidad Complutense de Madrid. Campus de Somosaguas s/n, Pozuelo de Alarcón, Madrid, Spain, **2** IBIMA, Unidad de Gestión Clínica de Salud Mental, Hospital Regional Universitario de Málaga, Universidad de Málaga, Málaga, Spain, **3** Institute of Physiological Chemistry, University Medical Center of the Johannes Gutenberg University of Mainz, Mainz, Germany, **4** Departamento de Biología Celular, Genética y Fisiología. IBIMA. Facultad de Ciencias, Universidad de Malaga. Campus de Teatinos s/n, Malaga, Spain

‡ These authors are shared senior authors on this work
* fernando.rodriguez@ibima.eu (FRF); rgomezhe@psi.ucm.es (RGH)

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Abstract

Undernutrition during pregnancy has been associated to increased vulnerability to develop metabolic and behavior alterations later in life. The endocannabinoid system might play an important role in these processes. Therefore, we investigated the effects of a moderate maternal calorie-restricted diet on the levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG), arachidonic acid (AA) and the N-acylethanolamines (NAEs) anandamide (AEA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) in the brain of newborn rat offspring. We focused on brain structures involved in metabolism, feeding behavior, as well as emotional and cognitive responses. Female Wistar rats were assigned during the entire pregnancy to either control diet (C) or restriction diet (R), consisting of a 20% calorie-restricted diet. Weight gain and caloric intake of rat dams were monitored and birth outcomes were assessed. 2-AG, AA and NAE levels were measured in hypothalamus, hippocampus and olfactory bulb of the offspring. R dams displayed lower gain weight from the middle pregnancy and consumed less calories during the entire pregnancy. Offspring from R dams were underweight at birth, but litter size was unaffected. In hypothalamus, R male offspring displayed decreased levels of AA and OEA, with no change in the levels of the endocannabinoids 2-AG and AEA. R female exhibited decreased 2-AG and PEA levels. The opposite was found in the hippocampus, where R male displayed increased 2-AG and AA levels, and R female exhibited elevated levels of AEA, AA and PEA. In the olfactory bulb, only R female presented decreased levels of AEA, AA and PEA. Therefore, a moderate diet restriction during the entire pregnancy alters differentially the endocannabinoids

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and/or endocannabinoid-related lipids in hypothalamus and hippocampus of the underweight offspring, similarly in both sexes, whereas sex-specific alterations occur in the olfactory bulb. Consequently, endocannabinoid and endocannabinoid-related lipid signaling alterations might be involved in the long-term and sexual dimorphism effects commonly observed after undernutrition and low birth weight.

Introduction

Decades ago, Barker and colleagues demonstrated a strong and paradoxical correlation between low birth weight and the development of metabolic syndrome in adulthood [1]. Simultaneously, Dutch Famine cohort studies showed the long-lasting and deleterious effects of undernutrition during early development [2]. These investigations led to propose the DOHaD (Developmental origin of Health and Disease) hypothesis, stating that early life insults could lead to increased vulnerability to develop diseases later in life [1] through a process known as programming [3]. Extensive investigations in this area have focused on the effects of undernutrition in the fetal period. Particularly, it has been shown that poor nutritional environment in pregnancy is commonly associated to low birth weight, and to the development of metabolic diseases, such as obesity and metabolic syndrome [1], whose prevalence is reaching epidemic proportions worldwide [4].

Currently, although overnutrition is much more common in developed countries, the consequences of undernutrition in critical windows of development represent still a burden. For instance, for women in rich societies, the pressure of being fit and thin may lead to gain less weight than recommended, increasing the risk to deliver a baby small for his gestational age [5, 6]. Similarly, women with a past of eating disorders are at high risk for suffering preterm birth and intrauterine growth restriction fetuses [7]. Despite the risk of metabolic diseases, underweight at birth has been associated to behavioral abnormalities, including alterations in cognitive performance, inadequate emotional responses or modifications in feeding behavior [8–10]. Therefore, this evidence emphasizes the importance to approach the burden of fetal undernutrition from different perspectives.

The effects of malnutrition during critical windows of human development by using animal models mostly focused on investigating metabolic and/or behavioral alterations [11–14]. Similarly to human studies, investigations using different animal species, but predominantly rodents, have demonstrated that the phenotype exhibited by offspring following undernutrition in utero may depend on the sex [11, 15–17] but also on the developmental stage where undernutrition occurs [2, 18–20]. Furthermore and importantly, the research using animal models has highlighted the underlying mechanisms leading to inadequate programming, showing alterations in brain structures involved in metabolism, learning and emotional processes after exposure to fetal undernutrition. For instance, the impairment of hypothalamic circuitry development, intimately connected to modifications in leptin signaling, has been described in animal models of intrauterine growth restriction [21, 22]. Moreover, dysregulation in hippocampal circuitries associated to BDNF (brain-derived neurotrophic factor) alterations in specific developmental stages has also been reported in offspring, either after exposure to a maternal calorie-restricted diet [23] or low dietary intake of n-3 polyunsaturated fatty acids (PUFAs) during pregnancy and lactation [24].

Closely related to leptin signaling and BDNF [25, 26], the endocannabinod system (ECS), a lipid signaling system, has emerged as a putative modulator of the biological mechanisms involved in developmental programming [27]. Indeed, the ECS has been demonstrated to be

crucial for regulating energy balance and food intake via central and peripheral mechanisms [28], as well as for the control of emotional responses and learning [29]. Consequently, ECS dysregulation has been associated to the development of obesity, metabolic syndrome and neuropsychiatric disorders [30, 31], which are diseases that might occur as a result of inadequate early life programming [1, 2, 8–13, 15–17, 20], as mentioned above. In addition to the endocannabinoids, non-cannabinoid acylethanolamines (OEA, PEA) that shares biosynthetic and degradation enzymatic pathways with anandamide, also contribute to the control of appetite, weight gain and lipid metabolism [28, 31, 32]

The main endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesized from phospholipids containing arachidonic acid (AA), which is a linoleic acid derivative [32] and belongs to the n-6 polyunsaturated fatty acid (PUFA) family. Noteworthy, several investigations have revealed the importance of PUFAs in brain development. For instance, a negative correlation between n-3 PUFA intake and increased vulnerability to neuropsychiatric disorders has been shown [33–36]. The role of n-6 PUFAs, precursors of arachidonic acid and ultimately of endocannabinoids, has been pointed out as critical in these processes as well [33, 37]. Specifically, endocannabinoid signaling plays a crucial role in important processes involved in brain maturation, including the establishment of adequate neural connections and synaptogenesis [33, 38]. Moreover, prenatal administration either of agonists, such as THC (Δ^9-tetrahydrocannabinol), or antagonists of cannabinoid receptors, has been associated to disruption of neuronal activity, defective establishment of cortical connectivity and behavioral abnormalities [39–43].

Although less investigated, altered nutritional conditions during early life might also have an impact on endocannabinoid signaling, leading to disturbances in brain functions and/or behaviors. Thus, prenatal and postnatal exposure to restricted omega-3 diet has been associated to impaired endocannabinoid-mediated neuronal functions in the adult brain, together with behavioral abnormalities [36]. Moreover, exposure to a maternal diet rich in n-3 or n-6 fatty acids modifies arachidonic acid and/or endocannabinoid levels in neonatal hypothalamus and hippocampus, resulting in alterations in the hypothalamus- pituitary-adrenal axis functions [44]. Therefore, this piece of evidence suggests that an inadequate endocannabinoid signaling resulting from exposure to an unbalanced maternal diet, might disrupt the establishment of functional circuitries involved in metabolism, learning and emotional control, leading to metabolic and neurobehavioral abnormalities later in life [27].

To date, only a few studies have addressed the relation between a global undernutrition in early life and endocannabinoid signaling. For instance, a pioneer study demonstrated modifications in the levels of endocannabinoids at weaning after maternal exposure to a calorie-restricted diet during pregnancy and/or lactation [45]. However, the impact of nutrient deficiency in earlier stages has been poorly investigated. Addressing this question could be especially pertinent considering that endocannabinoid levels fluctuate strongly during early development [46], which suggests a potentially critical contribution of endocannabinoid signaling in the earliest neural development processes. Accordingly, we have recently showed that exposure to a hypocaloric maternal diet implemented before and during gestation has an impact on the brain endocannabinoids and endocannabinoid-related lipids, leading to long-lasting consequences in offspring [47].

Taking into account that the timing of caloric restriction could be critical on the effects exhibited by offspring [2, 18–20] and that these effects might be sex-dependent [11, 15–17], this study aims at investigating the impact of a moderate caloric restriction applied during the entire pregnancy on male and female newborn rats. Particularly, we measured at birth the endocannabinoid, arachidonic acid and N-acylethanolamines (NAEs) content in brain structures involved in the modulation of metabolism, feeding behavior, learning and emotional responses, such as hypothalamus, hippocampus and olfactory bulb [28, 29, 48]. We hypothesize that endocannabinoid

signaling could be impaired in the offspring after exposure to maternal undernutrition during the complete pregnancy in a sex specific-manner.

Material and methods

This study was approved by the Animal Ethics Committee of the Complutense University of Madrid and was conducted in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and according to the Spanish regulations (RD 53/2013 and 178/2004).

Animals, diets and experimental design

Adult female Wistar rats (6 months old) (Harlan, Barcelona, Spain) were allowed to acclimate for a minimum of four weeks before the beginning of the experiments. Rats were handled and housed in groups under a 12 hours light-dark cycle with temperature of 22±1˚C. After the acclimation period, animals weighed 304±4 g and estrous cycle was evaluated daily. In the morning of proestrous, females were allowed to mate with a male of the same strain. Each male rat was mated with females from both groups (described below). The mating phase lasted 24 hours and occurred in the female cage. In the following morning the presence of vaginal plug or spermatozoa in vaginal smear confirmed successful mating, and this was defined as gestational day 0. Then, females rats were individually housed and randomly assigned to control (n = 4) or caloric restriction diet (n = 7) groups. At this stage, no statistical significant difference in body weight between groups was found.

Control rats (n = 4) were given free access to standard chow (Standard chow SAFE A04, Panlab, Barcelona, Spain). The standard chow provided 16.1% protein, 60% carbohydrate, 3.1% fat, 4% fiber, 0.0025% sodium and 2.9 kcal/g as energy content. In contrast, calorie-restricted dams (n = 7) were given a daily amount of food corresponding to 80% of the calories provided to control rats in the same gestational day, according to body weight (20% of caloric restriction). Water was provided ad libitum in both animal groups.

The day the dams were found with their respective litter was defined as postnatal 0 (PN0). Within 14 hours after birth, pups were weighed, sexed and sacrificed by quick decapitation. Brains were collected and brain regions were dissected for endocannabinoids measurement. None of the animals utilized in the present study showed signs of illness or died prior to the experimental endpoint.

Endocannabinoids and endocannabinoid related-lipids measurement

At PN0, male offspring chosen to be sacrificed were decapitated during the second/third hour of the dark phase and brains were quickly removed and frozen at −80˚C until brain region dissection. To avoid the possibility of variable outcomes among litters, brains from at least three litters per group were used to carry out endocannabinoids and endocannabinoid related lipid measurement (control male pups, n = 14-14-12 and male pups from calorie-restricted group, n = 14-18-14, for hypothalamus, hippocampus and olfactory bulb respectively; control female pups, n = 10-9-9 and female pups from calorie-restricted group, n = 10-10-10, for hypothalamus, hippocampus and olfactory bulb respectively). For the isolation of the selected brain regions, brains were thawed in cold Tris-HCl buffer (50 mM, pH = 7.4) and the entire hypothalamus, right hippocampus and right olfactory bulb was quickly dissected and immediately frozen at −80˚C until lipid extraction. The overall dissection procedure was carried out in less than 7 minutes for all animals to allow reliable comparative assessment of endocannabinoid levels.

For lipid extraction, pre-cooled steel balls of 5 mm were added to pre-cooled tubes containing the tissue. A solution of deuterated endocannabinoids (AEA-d4, 2-AG-d5, AA-d8, MAEA,

OEA-d2, PEA-d4 and 1-AG-d5, Cayman Chemicals, Ann Arbor, MI, USA) in acetonitrile was added to the tissue along with 300 μL of ice-cold 0.1 M formic acid and 300 μl of ethylacetate/hexane (9:1, v/v). Then, the samples were homogenized with a TissueLyser II (Qiagen, Hilden, Germany) for 60 s at 30 Hz. Subsequently, the samples were centrifuged for 10 min at 5,000 g and 4˚C and frozen at -20˚C for 20 min. The organic phase was removed and evaporated under a gentle stream of nitrogen at 37˚C. The aqueous phase was further used for protein content determination. The lipid extract was resolubilized in 50-μL acetonitrile/water (1:1, v/v) and quantitative analysis of the endocannabinoid levels was carried out by liquid chromatography-multiple reaction monitoring (LC-MRM). The concentrations of internal standards, as well as the calibration curves, were set and tailored using test hypothalamus, hippocampal and olfactory bulb tissues. The LC/MRM conditions for quantitative analysis of the endocannabinoids were set as previously described [49]. For protein quantification, the BCA method (bicinchoninic acid assay) was used and measurements were performed by using a FLUOstar Galaxy (BMG Labtechnologies).The endocannabinoid levels determined by LC/MRM were then normalized to the corresponding protein content of the tissues as previously described [49–51].

Statistical analysis

Caloric intake and body weight gain over time of rat dams were analyzed by two-way repeated measures analysis of variance (ANOVA), with time and pregnancy diet as factors. Multiple comparisons were assessed by Bonferroni post hoc test. Further analysis were performed by using the Student's *t*-test, when data passed the normality requirements (D'Agostini Pearson test), or Mann-Whitney's U test. A *p*-value below 0.05 was considered statistically significant.

Results

Impact of a moderate caloric restriction during gestation on rat dams

Effect of a moderate gestational restriction diet on maternal weight gain. Repeated measures ANOVA showed decreased cumulated weight gain in calorie-restricted dams as compared to controls during the entire pregnancy ($F_{(1,9)} = 5.7$, $p<0.05$). Specifically, Bonferroni multiple comparisons showed that statistically significant differences between groups started at gestational day 12 ($F_{(1,9)} = 7.78$, $p<0.05$) and lasted up to day 20 ($F_{(1,9)} = 6.40$, $p<0.05$) (**Fig 1A** and **S1 Data**). Moreover, at PN0, calorie-restricted mothers weighed significantly less than controls (mean weight and SEMs of controls vs calorie-restriction: 342.1 9.38 vs 303.2 9.82, Mann-Whitney's U test, $U(4,7) = 3$, $p<0.05$) (data not shown).

Effect of a moderate gestational restriction diet on maternal caloric intake. According to the experimental design carried out, the cumulative caloric intake of calorie-restricted dams was decreased (repeated measures ANOVA, $F_{(1,9)} = 184.51$, $p<0.001$). Statistically significant differences between groups started at gestational day 1 ($F_{(1,9)} = 53.08$, $p<0.001$) and lasted up to the end of measurements (day 20) ($F_{(1,9)} = 169.53$, $p<0.001$) (**Fig 1B**).

Taken together these data indicate that calorie-restricted diet has an impact on weight gain and absolute body weight during pregnancy. Moreover, taking into account the experimental design adopted in the present study, calorie-restricted dams consumed less calories as compared to controls.

Effect of a moderate maternal caloric restriction on birth outcomes

Pups from control dams and calorie-restricted mothers were born between gestational day 21–22. At birth, offspring from gestational calorie-restricted dams weighed significantly less

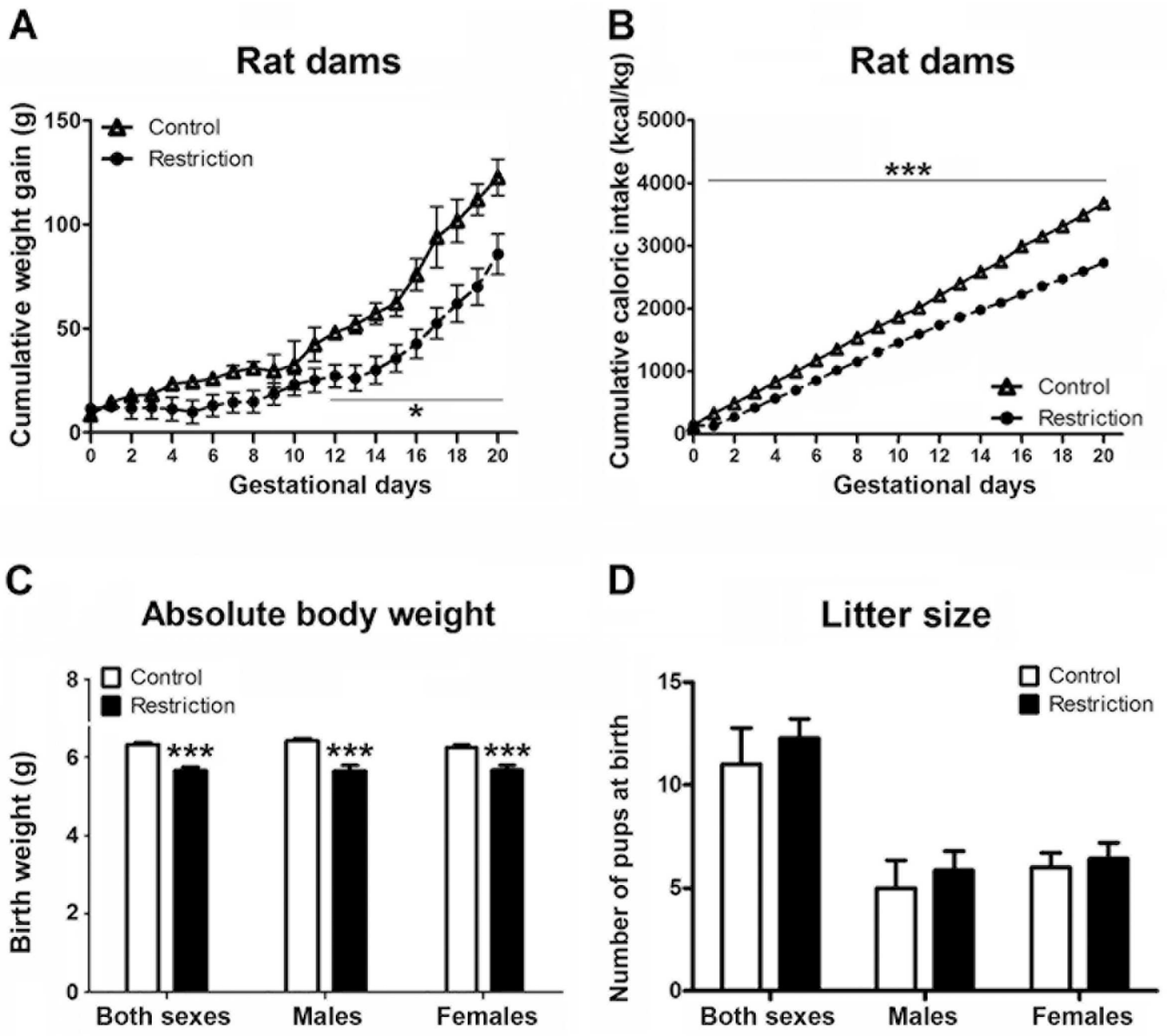

Fig 1. Effect of gestational calorie-restriction on rat dams and birth outcomes. Experiments started the following day of mating. Calorie-restricted rat dams (n = 7) received 80% of control dams (n = 4) food intake according to body weight, which was measured daily (restriction of 20%). Calorie-restricted diet lasted until birth. Figures A and B describe the cumulative weight gain (g) and cumulative caloric intake (Kcal/Kg), respectively, of control (open triangles) and calorie-restricted (solid circles) dams during pregnancy. At PN0 (birth day), litter size was evaluated and pups were sexed and weighed. Figures C and D describe the absolute body weight (g) and litter size, respectively, of offspring from control dams (n = 30) and offspring from calorie-restricted dams (n = 47) at birth (open and solid bars, respectively). Values are expressed as mean +/- SEM. Data were analyzed with repeated measures ANOVA followed by Bonferroni multiple comparisons (A, B), and Student´*t* test (C, D): **p*<0.05, ****p*<0.001.

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than controls: both sexes taken together (t = 6.199, p<0.001); male (t = 4.768, p<0.001); female (t = 3.997, p<0.001) (Fig 1C). In contrast, no significant differences in litter size were found either in both sexes analyzed together or in each sex analyzed separately (Fig 1D). Thus, gestational calorie-restriction leads to underweight at birth without modifying the litter size.

Impact of a moderate maternal caloric restriction on endocannabinoid and endocannabinoid-related lipid levels in specific brain regions of male and female offspring at birth

Hypothalamic endocannabinoid and endocannabinoid-related lipid levels in male and female offspring at birth. Statistically significant differences between perinatal groups were found in endocannabinoids and/or endocannabinoid-related lipids at birth in male and female offspring. Specifically, male pups from gestational calorie-restricted dams displayed significant lowers levels of AA as compared to controls ($U = 45.00$, $p<0.05$) (Fig 2C and S2 Data), but similar levels of AEA ($t = 0.8515$, $p>0.05$) and 2-AG ($t = 1.275$, $p>0.05$) (Fig 2A and 2B, respectively). Regarding NAEs levels, offspring from calorie-restricted dams presented lower concentrations of oleoylethanolamide (OEA) ($U = 46.00$, $p<0.05$) (Fig 2D), but no significant differences in palmitoylethanolamide (PEA) levels ($U = 79$, $p>0.05$) (Fig 2E). Female pups exhibited decreased level of 2-AG ($t = 2.649$, $p<0.05$) (Fig 3B) but no differences either in AEA or AA ($U = 42$, $p>0.05$ and $U = 36$, respectively) were found (Fig 3A and 3C). Females also presented a reduction of PEA levels ($t = 2.197$, $p<0.05$) (Fig 3D). The OEA values in the hypothalamus could not be reliably quantified in female offspring (data not shown).

Taken together, these data show that a moderate caloric restriction diet during pregnancy decreases hypothalamic content of the endocannabinoid and/or the endocannabinoid-related lipids in the offspring with sex-dependent differences.

Hippocampal endocannabinoid and endocannabinoid related-lipid levels in male and female offspring at birth. Measurements of hippocampal endocannabinoid and endocannabinoid-related lipids showed statistical differences between perinatal groups in both sexes.

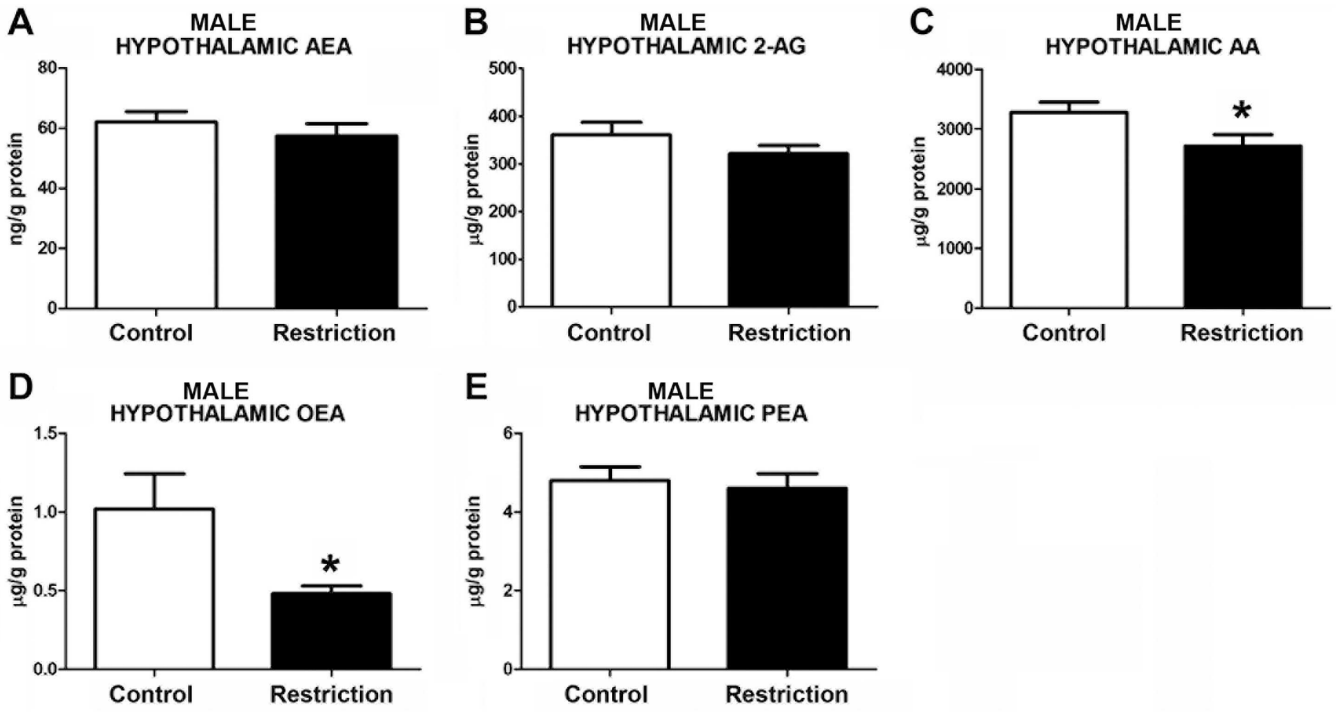

Fig 2. Endocannabinoid and endocannabinoid-related lipid levels in the hypothalamus of male offspring at birth. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonic acid (AA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) levels in the hypothalamus of male offspring (A-E) from control dams (n = 14) and calorie-restricted dams (n = 14) at birth (open bars and solid bars, respectively). Values are expressed as mean +/- SEM. Data were analyzed by Student´s *t*-test (A, B) or Mann Whitney´s U test (C, D, E): *$p<0.05$.

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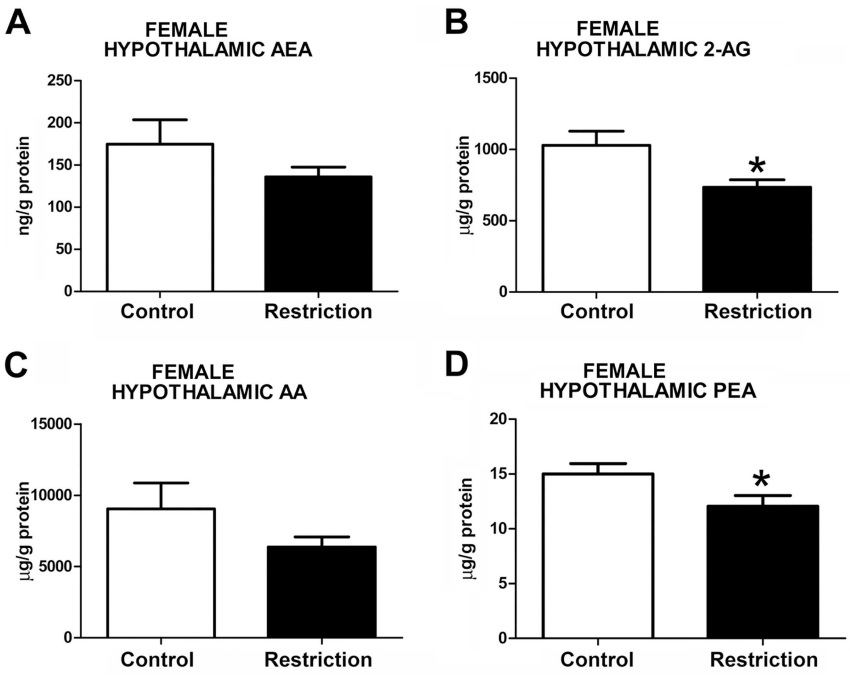

Fig 3. Endocannabinoid and endocannabinoid-related lipid levels in the hypothalamus of female offspring at birth. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonic acid (AA) and palmitoylethanolamide (PEA) levels in the hypothalamus of female offspring (A-D) from control dams (n = 10) and calorie-restricted dams (n = 10) at birth (open bars and solid bars, respectively). Values are expressed as mean +/- SEM. Data were analyzed by Student´s *t*-test (B, D) or Mann Whitney´s U test (A, C): **p*<0.05.

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Specifically, the male offspring from calorie-restricted dams displayed increased levels of 2-AG ($t = 2.721$, $p<0.05$) and AA ($U = 65.00$, $p<0.05$) (Fig 4B and 4C, respectively, and S3 Data). A strong tendency to increased levels of PEA in calorie-restricted male offspring was also detected ($t = 1.775$, $p = 0.08$) (Fig 4E). However, no differences between groups either in AEA ($t = 0.1325$, $p>0.05$) or OEA ($U = 93$, $p>0.05$) levels were found (Fig 4A and 4D, respectively). In contrast, calorie-restricted female pups showed increased hippocampal AEA ($t = 2.264$, $p<0.05$) (Fig 5A) and, similarly to male offspring, enhanced levels of AA ($t = 2.401$, $p<0.05$) (Fig 5C), although 2-AG levels were unchanged ($t = 1.489$, $p>0.05$) (Fig 5B). Moreover, female offspring from diet-restricted dams presented higher hippocampal levels of PEA ($U = 18$, $p<0.05$) than control female pups (Fig 5D). The OEA values in the hippocampus could not be reliably quantified in female offspring (data not shown).

Taken together, these data indicate that a moderate caloric restriction during pregnancy increases the hippocampal endocannabinoids and/or endocannabinoid-related lipids in the offspring with sex-dependent differences.

Endocannabinoid and endocannabinoid-related lipid levels in the olfactory bulb of male and female offspring at birth. The statistical analysis did not reveal any alteration in endocannabinoids, such as AEA ($t = 0.68$, $p>0.05$) and 2-AG ($U = 75$, $p>0.05$), AA ($U = 68$, $p>0.05$) and PEA ($U = 68$, $p>0.05$) levels in the olfactory bulb of male offspring from calorie-restricted dams as compared to controls (Fig 6). In contrast, significant alterations in the endocannabinoid and endocannabinoid-related lipids were detected in female restricted offspring (Fig 7 and S4 Data). Specifically, calorie-restricted females exhibited decreased levels of AEA ($t = 3.279$, $p<0.01$) (Fig 7A), AA ($t = 2.471$, $p<0.05$) (Fig 7C) and PEA ($t = 2.639$, $p<0.05$) (Fig 7D) in this brain structure. No differences were found between groups in the levels of

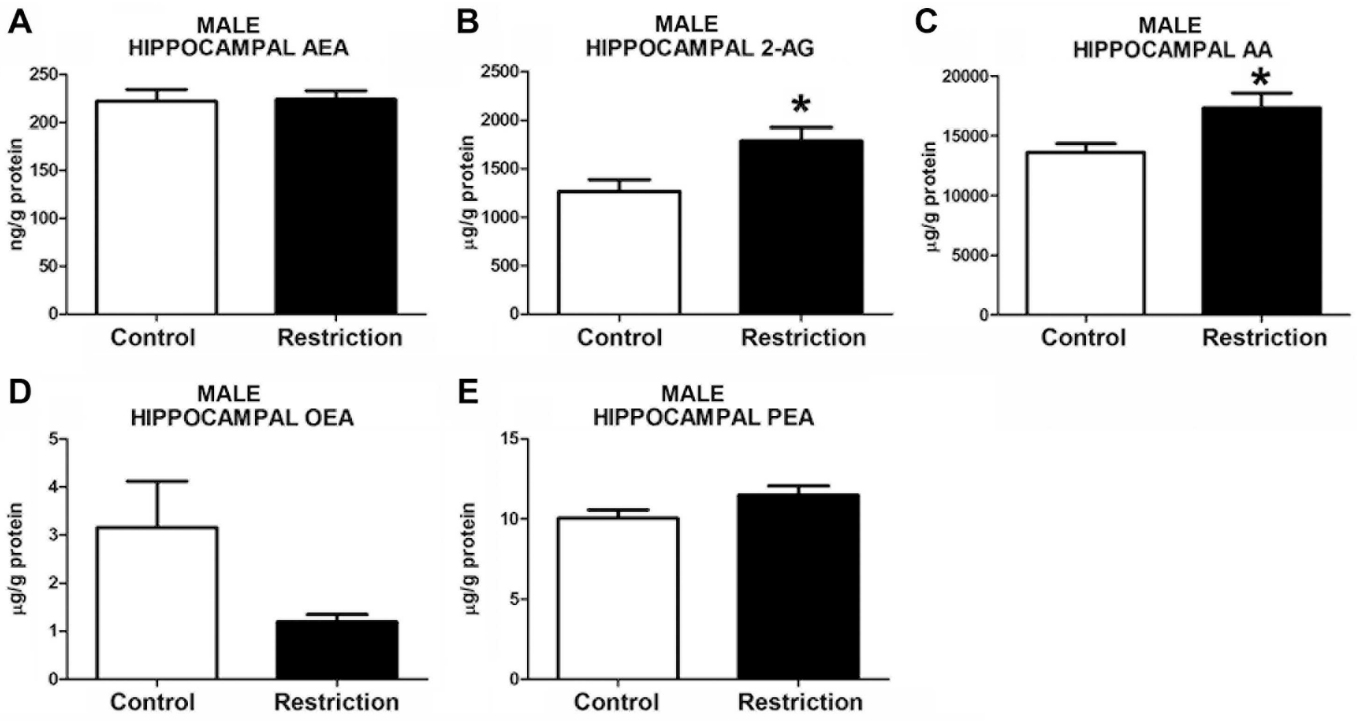

Fig 4. Endocannabinoid and endocannabinoid-related lipid levels in the hippocampus of male offspring at birth. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonic acid (AA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) levels in the hippocampus of male offspring (A-E) from control dams (n = 14) and calorie-restricted dams (n = 18) at birth (open bars and solid bars, respectively). Values are expressed as mean +/- SEM. Data were analyzed by Student´s *t*-test (A, B, E) or Mann Whitney´s U test (C, D): **p*<0.05

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2-AG (t = 1.550, p<0.05) (Fig 7B). The OEA values in the olfactory bulb could not be reliably quantified in both male and female offspring (data not shown).

Taken together, these data indicate that a moderate caloric restriction diet during pregnancy modifies the levels of endocannabinoids and/or endocannabinoid-related lipids in the hypothalamus, hippocampus and/or olfactory bulb of offspring. Specifically, male and female offspring from calorie-restricted dams that were underweight at birth displayed decreased endocannabinoids and endocannabinoids-related lipids in the hypothalamus, whereas the opposite was found in the hippocampus. The female offspring also showed the same tendency as hypothalamus to reduced endocannabinoid and endocannabinoid-related lipids in the olfactory bulb. Importantly, alterations in each endocannabinoid and/or related lipid occurred differently according to the sex of the offspring.

Discussion

The main finding of the present study is that newborn rats exposed to a moderate caloric restriction during the entire pregnancy displayed alterations in endocannabinoids and/or endocannabinoid-related lipids, in brain structures involved in the regulation of metabolism and emotional and cognitive responses. Specifically, male and female offspring from diet-restricted dams exhibited decreased levels of the main endocannabinoids, their precursor and/or NAEs in the hypothalamus and, conversely, they showed enhanced content of these lipids in the hippocampus. This similar profile between males and females from calorie-restricted dams was not evident in the olfactory bulb, where the calorie-restricted female offspring presented decreased levels of AEA, their precursor (AA) and PEA. Moreover, these modifications

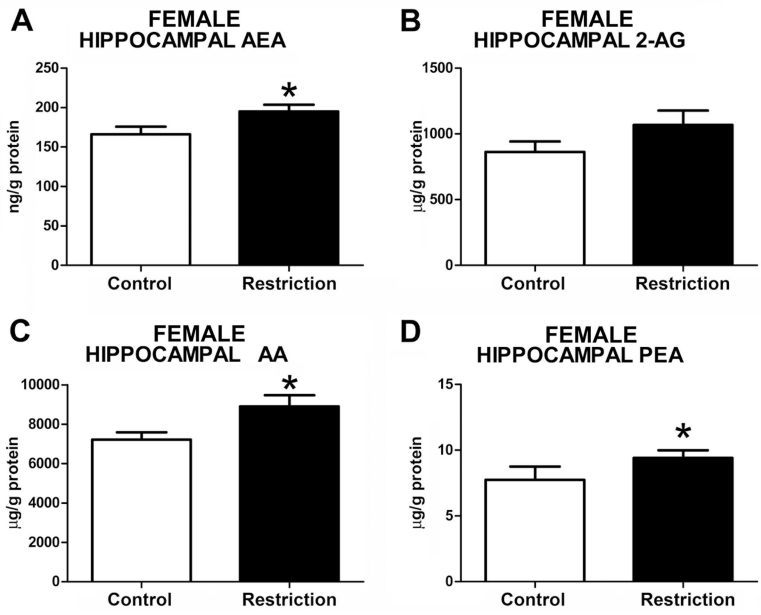

Fig 5. Endocannabinoid and endocannabinoid-related lipid levels in the hippocampus of female offspring at birth. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonic acid (AA), and palmitoylethanolamide (PEA) levels in the hippocampus of female offspring (A-D) from control dams (n = 9) and calorie-restricted dams (n = 10) at birth (open bars and solid bars, respectively). Values are expressed as mean +/- SEM. Data were analyzed by Student´s *t*-test (A, B,C) or Mann Whitney´s U test (D): *$p<0.05$.

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were accompanied of underweight at birth, a common result when diet restriction is applied for the entire pregnancy or in the last phases of pregnancy [12, 13]. Interestingly, this finding has been widely associated to metabolic and behavioral abnormalities later in life [12–14].

The majority of animal research conducted to evaluate the effects of undernutrition in critical developmental windows has focused on investigating the deleterious effects of a severe gestational diet restriction in the offspring [12, 13]. In our study, we have adopted a moderate maternal calorie-restriction, that may have simulated better the reduction in food intake documented in some human studies, commonly associated to decreased weight gain during pregnancy [5, 6, 52], and could have prevented unnecessary effects in the animals. In rodents, this type of maternal restriction has been demonstrated previously to be enough to induce long-lasting alterations in offspring [11, 14, 53, 54]. Moreover, we have recently showed that the preconceptional and gestational exposure to a moderate calorie-restricted diet increases the risk of developing features of metabolic syndrome as well as behavioral abnormalities in the offspring [47, 55]. Importantly, in the present study, we have demonstrated for the first time that a moderate maternal caloric restriction applied during the entire pregnancy alters brain endocannabinoid and/or endocannabinoid-related lipid levels at birth in male and female offspring and reduces the weight at birth.

The modifications in the supply of nutrients to the fetus may have altered the intrauterine growth leading to inadequate size at birth in our study. Regarding the most important nutrients during intrauterine life, apart from glucose and aminoacids, the fatty acids and, particularly, the long-chain (LC) PUFAs, such as the arachidonic acid (AA) and docosahexaenoic acid (DHA) has been revealed as critical elements for a correct growth and neurodevelopment [37, 56]. The concentration of LC-PUFAs and their precursor depends on diet, fatty acid storage in the adipose tissue and endogenous synthesis, which requires adequate functionality of the enzymes involved in desaturation and elongation of essential fatty acids [57]. Therefore, to

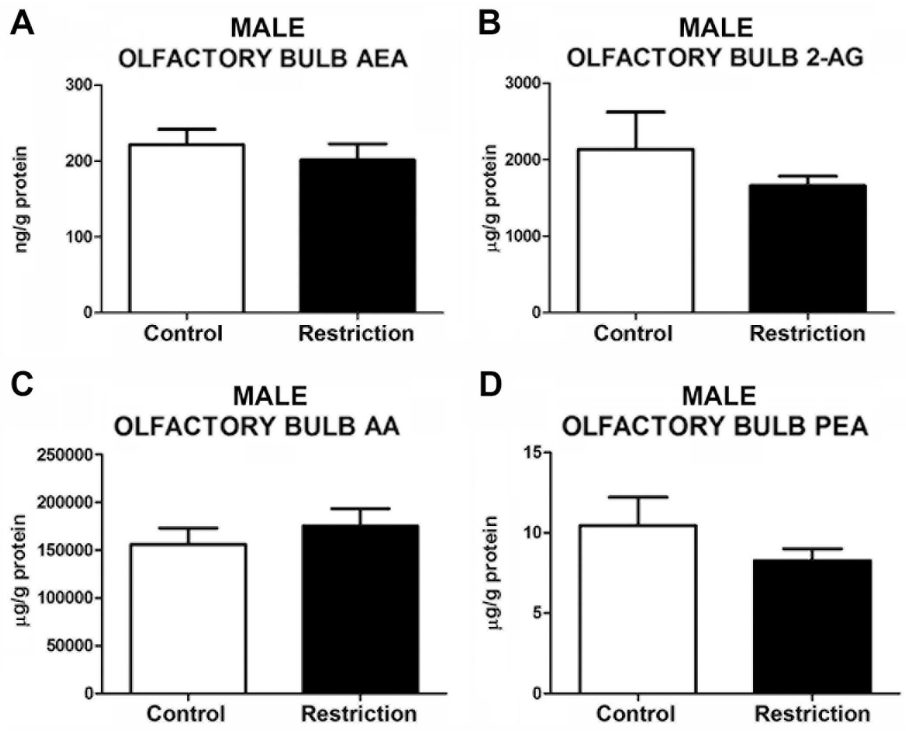

Fig 6. Endocannabinoid and endocannabinoid-related lipid levels in the olfactory bulb of male offspring at birth. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonic acid (AA) and palmitoylethanolamide (PEA) levels in the olfactory bulb of male offspring (A-D) from control dams (n = 12) and calorie-restricted dams (n = 14) at birth (open bars and solid bars, respectively). Values are expressed as mean +/- SEM. Data were analyzed by Student´s *t*-test (A) or Mann Whitney´s U test (B, C, D): **p*<0.05.

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ensure an adequate fatty acid supply, the fetuses and new born animals depend on the mother nutritional status and the placenta functionality to obtain both essential fatty acids (EFAs) and long-chain fatty acids (LC-PUFAs). This is due to the limited capacity of the fetus to transform EFAs and the importance of depositing the PUFAs in key tissues, including the fetal brain, during intrauterine growth [57, 58]. Regarding the endocannabinoids, little is known about the fetal-maternal relationship in endocannabinoid content. It has been proposed that, although n-6 PUFA derivatives may be synthetized by the fetus in the tissues, the placenta may also transfer maternal endocannabinoids to the fetus by contributing to maintenance of the endocannabinoid basal tone [27]. Consequently, a maternal caloric restriction in our study may have had an impact on the levels of PUFAs and their derivatives, including the endocannabinoids, in the fetus, and leading to alterations in endocannabinoid and related lipid levels in different brain structures.

Indeed, we found decreased levels of endocannabinoids and/or endocannabinoid-related lipids in the hypothalamus of male and female offspring. Specifically, we found decreased AA levels in the hypothalamus of male offspring, without any change in the concentrations of AA-derived endocannabinoids (i.e., AEA and 2-AG). In contrast, the female offspring exhibited decreased levels of 2-AG in this brain region despite the unchanged concentrations of its precursor (AA). Apart from the reduced concentration of the LC-PUFAs after a caloric restriction, including the reduction of the AA, precursor of endocannabinoids, an alternative explanation to these findings may involve a sex-specific alteration in the activity and/or levels of the endocannabinoid metabolic enzymes. Therefore, in an attempt to maintain endocannabinoid and/or

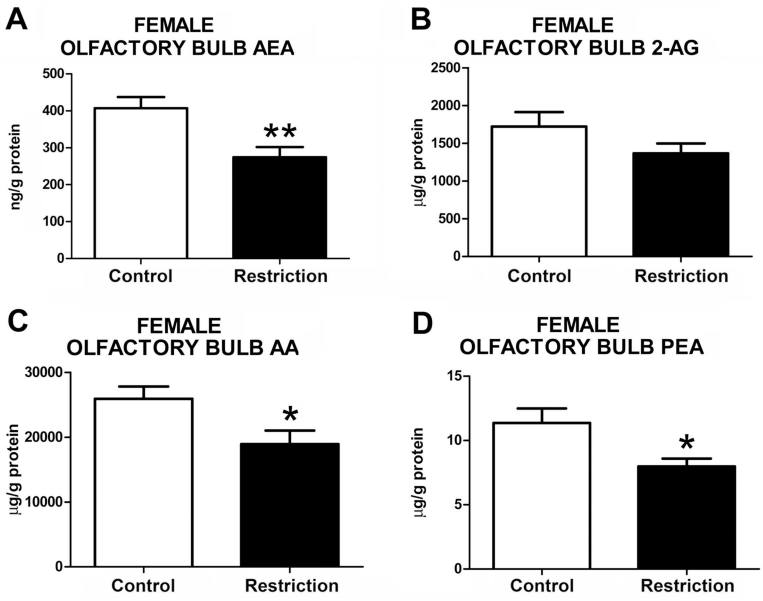

Fig 7. Endocannabinoid and endocannabinoid-related lipid levels in the olfactory bulb of female offspring at birth. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), arachidonic acid (AA) and palmitoylethanolamide (PEA) levels in the olfactory bulb of female offspring (A-D) from control dams (n = 9) and calorie-restricted dams (n = 10) at birth (open bars and solid bars, respectively).Values are expressed as mean +/- SEM. Data were analyzed by Student´s *t*-test: **p*<0.05, ***p*<0.01.

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AA homeostasis, an increased synthesis of 2-AG and AEA could have lately determined the reduction of AA in male offspring. Conversely, an increased degradation of 2-AG may have determined its reduction and maintained the AA content in females unchanged. Further analysis of the expression and activity of enzymes responsible for biosynthesis and degradation of endocannabinoids has to be addressed to confirm this hypothesis. Another possibility is that an increased endocannabinoid transfer from mother, through placenta, might have also contributed to maintain an endocannabinoid tone in the hypothalamus of male offspring [27], despite the decreased AA availability and the depletion in maternal tissues due to undernutrition. This idea is supported by previous studies showing differences in male and females placentas after inadequate maternal diets [59, 60].

Our finding in male hypothalamus contrasts with a previous report [45], describing decreased hypothalamic endocannabinoid levels in male offspring coming from dams exposed to a 50% calorie-restricted diet during late pregnancy. However, in this interesting study and differently to ours, the endocannabinoid measurement was performed at weaning, and not at birth. Considering that the pups are independent from the maternal fatty acid stores at this developmental stage, the levels of endocannabinoids here may easily have reflected the metabolic status of the pups and, thus, their own fatty acid stores. Specifically, in this previous study the pups at weaning exhibited decreased body weight that was accompanied by a reduction of hypothalamic AEA. Although the body weight at birth was not reported in this study, similar types of restriction have been associated to decreased body weight at birth, which suggests that these animals did not exhibited a rapid catch-up growth. This phenomenon has been described after caloric restriction in pregnancy and/or small fetuses associated to hyperphagia [12, 13] and is known to induce a severely dysfunctional metabolic phenotype in the offspring later life [13, 61]. Therefore, the decreased hypothalamic anandamide described in the study of Matias et al. (2003) might have affected the appetite [62, 63] in these animals, leading to a lean

phenotype at weaning and favoring complete recovery at adulthood. Although we did not evaluate the long-term effects on the offspring, the opposite might have occurred in our study. Particularly, we found decreased levels of OEA in the hypothalamus of male offspring from calorie-restricted mothers. Considering that this NAE is involved in the modulation of satiety [64], our data suggest a possible increased appetite in these animals, similar to previously reported in offspring exposed to undernutrition in pregnancy and undergoing to rapid catch-up growth during lactation and infant period and impaired metabolic phenotype at adulthood [13, 61].

Moreover, in female hypothalamus we found decreased levels of PEA, a NAE with anti-inflammatory and antiobesity properties [65, 66], suggesting the possibility of inflammatory status in female offspring, which has been associated to the development of metabolic and behavioral abnormalities [67–69].

The reduction of the levels of OEA and PEA in male and female offspring may be a consequence of a limited amount of the precursors required for their synthesis, particularly the oleic acid and/or palmitic acid, as previously proposed in adult animals [70, 71] and recently demonstrated in humans [72]. Additionally, the impairment of the activity and/or levels of the enzymes implicated in the synthesis and degradation of NAEs might also explain these results. Further research is needed to address these possibilities.

Concerning the possible interpretation to our findings in hypothalamus, it is important to note that previous studies have showed that the reduction in both AA and endocannabinoid levels in this brain structure at birth is associated to the development of metabolic disturbances at adulthood [47, 51]. Although alterations in endocannabinoids were not observed in male offspring in this brain region, the decreased levels of AA and their association with decreased birth at weight found in the present study might have promoted adverse consequences in the development of hypothalamus. This supposition takes into account that AA is a LC-PUFA involved in growth and brain development and is the precursor of the eicosanoids, which contribute to regulate cell proliferation, growth, immunity and inflammation [37].

We also evaluated the endocannabinoid levels in the hippocampus, a brain structure involved in modulating emotional responses and where the endocannabinoid system plays an important role in memory formation and neurogenesis associated to metabolism-dependent mechanisms [29, 73]. Intriguingly, we found increased hippocampal levels of AA and 2-AG at birth in male offspring from calorie-restricted dams, which is opposite to the findings in the hypothalamus. In the female hippocampus we also found increased concentration of AEA, AA and PEA levels. The increased endocannabinoid and AA levels found in our animals might reflect a fetal adaptation against the reduced availability of nutrients to preserve hippocampal development. This idea is supported by previous works showing fluctuating BDNF levels, a protein related to endocannabinoids [26], in different stages of the brain development of offspring from severely calorie-restricted dams [23]. Interestingly, a peak in 2-AG levels at PN1 in the whole brain has been described together with increased expression of the cannabinoid receptor type 1 (CB_1), suggesting the importance of endocannabinoids (i.e., 2-AG) and endocannabinoid synthesis precursors (i.e., AA) for an adequate brain development [46]. Considering this evidence, the increased 2-AG and AA levels at PN0 in male offspring may reflect a premature peak to prevent the deleterious effects on hippocampus development. In female offspring we found increased AEA and PEA levels. It is interesting to note that the enzyme fatty acid amide hydrolase (FAAH) degrades both AEA and PEA [74], which suggests that an eventual alteration in this metabolic enzyme may be implicated. Moreover, the increased levels of AA, 2-AG and AEA in male and female offspring suggest that implementing a moderate caloric restriction in rat previously well-nourished might have a modest impact on the fatty acid storage at the beginning of pregnancy, favoring the deposition of AA in hippocampus. In

particular, it is interesting to note that these findings contrasts with the results recently described by our group in offspring from mothers exposed to the same caloric restriction during the preconceptional and gestational period, and with presumably decreased maternal stores. In this previous study, we observed decreased levels of AEA in hippocampus at birth in association to increased anxiety-related responses in adolescence [47], suggesting that the potential compensatory effect of increased endocannabinoids in the hippocampus is inverted in worse nutritional conditions. Further research is needed to confirm these possibilities.

Although the role of the endocannabinoid system in behavioral programming has not been well established yet, alterations in hippocampal endocannabinoid content are known to promote impaired emotional and cognitive responses. For instance, a decrease in the hippocampal 2-AG level has been correlated to anxiety-related responses [51, 75, 76], and the blockade of anandamide reuptake specifically in the hippocampus produces anxiolytic effects [77]. Furthermore, increased 2-AG in hippocampus was associated to mitigation of the cognitive alterations in severely undernourished mice supplemented with a diet rich in fish oil, an important source of n-3 PUFAs [78], although the opposite has been described in an animal model of schizophrenia [79]. In the context of nutritional programming, emotional responses and cognitive performance have been found to be affected after exposure to undernutrition during critical windows of development and/or in new born small for gestational age [14, 20, 80] and in a sex specific-manner [81, 82]. Despite this evidence, the increased levels of 2-AG, AEA and AA we found in the hippocampus are difficult to interpret, considering that CB_1 receptor activation by endocannabinoids may mediate bimodal opposite responses depending on the differential distribution of CB_1 in distinct neuronal populations [29].

Additionally, we measured endocannabinoid and NAE levels in the olfactory bulb of male offspring. The contribution of the endocannabinoid system has been revealed recently in this brain structure, where CB_1 receptor stimulation increases odor perception and food intake in fasted animals [48]. In male offspring, we did not detect any modification in the endocannabinoid levels in the olfactory bulb from calorie-restricted dams, even though these animals displayed higher levels of OEA in the hypothalamus, which was probably associated to disrupted hunger and/or feeding behavior in these animals. However and interestingly, the female offspring displayed decreased levels of AEA, AA and PEA in the olfactory bulb, suggesting alterations in feeding behavior and an inflammatory status. The findings in female offspring are in agreement to a previous report showing that prenatal adverse conditions (such as prenatal stress) can affect odor preference in a sex specific-manner, leading to alterations in odor preference in female offspring [83] and suggesting that the females might have increased vulnerability in this brain structure after exposure to adverse perinatal conditions.

The changes in endocannabinoid and/or endocannabinoid-related lipid levels found in the hypothalamus and hippocampus of male and female offspring from calorie-restricted dams raise several questions. On the one hand, as modifications in these lipid regulators were found in developing brain structures in association with decreased weight at birth, we cannot discard the possibility that these alterations might have long-lasting consequences in the offspring, as it has been previously reported [12–14]. Indeed, alterations in endocannabinoid signaling in brain structures involved in the modulation of metabolism and emotional responses may lead to inadequate neuronal wiring or subtle alterations in neuronal connectivity and favor vulnerability to diseases later in life [27]. Moreover, previous studies have shown that alterations in endocannabinoid signaling during early development after treatment with specific agonists/antagonists of the CB_1 cannabinoid receptors are associated to long-lasting neurochemical, endocrine and behavioral effects [39–43]. In support of this notion, we have recently reported changes in brain endocannabinoids and endocannabinoid-related lipids at birth after inadequate maternal diets in hypothalamus and hippocampus in association with metabolic and

behavioral alterations [47, 51]. On the other hand, it is possible that some brain structures were protected from the effects of a moderate caloric restriction implemented only during pregnancy by the preferential uptake of the fetal tissues of specific LC-PUFAs, such as AA or n-3 PUFAs. It is interesting to note here that the n-3 PUFAs can affect the levels of endocannabinoids by decreasing their levels by competing for the metabolic enzymes [70, 84], or by increasing their levels depending on different circumstances [78, 85]. Additionally, the presence of sexual dimorphism mainly associated to the alterations found in olfactory bulb and the PEA levels in all the brain regions of females, suggest that the maternal calorie-restriction might have affected the male and female offspring through different mechanisms. Further investigations are needed to explore these possibilities.

Conclusions

In summary, we have demonstrated that a moderate caloric restriction during the entire pregnancy results in underweight offspring with altered endocannabinoid, AA and/or NAE levels in the hypothalamus, hippocampus and/or olfactory bulb of male and female offspring at birth in a sex-specific manner. These data represent a first step towards understanding the possible contribution of the ECS in the nutritional programming, considering the available data on the long-lasting effects of undernutrition and underweight at birth. Understanding why dietary manipulations modify hypothalamic, hippocampal and olfactory bulb endocannabinoid and endocannabinoid-related lipid levels, and whether these changes lead to permanent dysfunctions in the ECS and/or impairment in circuitries involved in the regulation of metabolism and emotional behaviors in a sex-specific manner need to be elucidated. Therefore, further investigations are required to clarify the role of the ECS in nutritional programming.

Supporting information

S1 Data. Gestation weight gain in rat dams.
(PZF)

S2 Data. Endocannabinoid and endocannabinoid-related lipid levels in hypothalamus.
(PZF)

S3 Data. Endocannabinoid and endocannabinoid-related lipid levels in hippocampus.
(PZF)

S4 Data. Endocannabinoid and endocannabinoid-related lipid levels in olfactory bulb.
(PZF)

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Author Contributions

Conceptualization: FRF LB BL RGH JS.

Formal analysis: LB MTRL JS.

Funding acquisition: FRF BL LB RGH JS.

Investigation: MTRL MV FA JD MA RNB RA EL CH LB LO JS.

Methodology: LB MTRL JS FRF BL.

Writing – original draft: FRF MTRL.

Writing – review & editing: BL JS EL LO RGH LB.

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