METHYL DONOR SUPPLEMENTATION IN RATS REVERSES THE DELETERIOUS EFFECT OF MATERNAL SEPARATION ON DEPRESSION-LIKE BEHAVIOUR

Running head: Methyl donor supplementation and maternal separation

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Abstract

Adverse early life events are associated with altered stress responsiveness and metabolic disturbances in the adult life. Dietary methyl donor supplementation could be able to reverse the negative effects of maternal separation by affecting DNA methylation in the brain. In this study, maternal separation during lactation reduced body weight gain in the female adult offspring without affecting food intake, and altered total and HDL-cholesterol levels. Also, maternal separation induced a cognitive deficit as measured by NORT and an increase in the immobility time in the Porsolt forced swimming test, consistent with increased depression-like behaviour. An 18-week dietary supplementation with methyl donors (choline, betaine, folate and vitamin B12) from postnatal day 60 also reduced body weight without affecting food intake. Some of the deleterious effects induced by maternal separation, such as the abnormal levels of total and HDL-cholesterol, but especially the depression-like behaviour as measured by the Porsolt test, were reversed by methyl donor supplementation. Also, the administration of methyl donors increased total DNA methylation (measured by immunohistochemistry) and affected the expression of insulin receptor in the hippocampus of the adult offspring. However, no changes were observed in the DNA methylation status of insulin receptor and corticotropin-releasing hormone (CRH) promoter regions in the hypothalamus. In summary, methyl donor supplementation reversed some of the deleterious effects of an early life-induced model of depression in rats and altered the DNA methylation profile in the brain.

Keywords: Porsolt, insulin receptor, stress, DNA methylation, cholesterol, Nort, hippocampus

1. Introduction

Epidemiological studies show that adverse early life events are associated with altered stress responsiveness and enhanced vulnerability to the development of psychopathologies like depression and anxiety disorders [1]. Moreover, postnatal environment is known to play a relevant role in neurodevelopment and behavioural responses in adulthood [2]. Thus, maternal separation (MS) is a well-known animal paradigm, which is emotionally stressful for the rodent pups [3], resulting in animals with behavioural and neuroendocrine signs of elevated stress reactivity in adulthood. MS is nowadays considered as a useful model to study depression [4,5].

Epigenetics is one of the key mechanisms involved in the development of the central nervous system, conferring tissue-specific gene expression to its three major cell types: neurons, astrocytes, and oligodendrocytes [6]. Previous research has hypothesized that the normal development and function of the central nervous system is dependent on the methylation of cytosines [7,8,9,10]. Thus, DNA-methyltransferases (DNMTs) and methyl-CpG-binding protein 2 (MeCP2) are highly expressed in developing brains but decreases later in life [10,11]. Mutations in MECP2 are involved in the neurodevelopmental disorder Rett syndrome, as well as in some cases of mental retardation and autism [7,9,12,13]. Mice lacking the gene *MBD2* (methyl-CpG-binding domain protein 2) show neurobehavioural manifestations, while mice lacking *DNMT1* only in the brain lose neurons and die after birth [8]. Acute stress has been demonstrated to affect both the global DNA methylation profile of the brain [14] and the methylation levels of genes implicated in the regulation of the hippocampal function, such as the glucocorticoid receptor [15]. Moreover, it has been described that early-life stress in rodents induces adult behavioural impairments [16] and leads to epigenetic alterations in different regions of the brain [17,18], affecting for example the methylation of the

promoters of reelin [15] and corticotropin-releasing factor [19] in the adult rat brain. It has been also described that these marks could be modified, even in terminally differentiated tissues, not only by pharmacological agents but also by stable variations in environmental conditions, such as the diet [20]. Thus, neuropsychiatric symptoms like fatigue and depression have been described in patients with folate deficiency, whereas studies in animals correlated folate-responsive depression with low levels of S-adenosylmethionine (SAM) [21]. Similar findings have been observed in vitamin B12 deficiency [22], suggesting that low levels of SAM, which can be influenced by dietary methyl donor intake, alter DNA methylation status in the brain.

Previous studies have reported nutrition as a factor that can modify the stress response [2,23]. Thus, Dallman *et al.* [24] characterized palatable food as a self-medication against stress signs. Krause *et al.* [25] reported that animals with elevated levels of sodium secreted fewer stress hormones and that they were more interactive and less socially anxious. Moreover, high levels of homocysteine, an intermediate in the methionine metabolic pathway, have been described as one of the causal factors for dementia, cognitive impairment [26,27] and Alzheimer's disease [28].

Based on this information and on previously reported beneficial effects of the methyl donor supplementation (MD) on hepatic triglyceride accumulation induced by an obesogenic diet [29], we hypothesized that a diet rich in methyl donors during adulthood could diminish the effect of adverse early life conditions in rats. The novelty of the current work lies in studying some of the epigenetic mechanisms that can be modulated by the diet (in this case, methyl donor supplementation) in order to counteract early life stress-induced depression-like behaviour. This approach could be very valuable for developing new nutritional strategies to combat early-life stress-induced neurological impairments. For this purpose, we have evaluated the central and

peripheral effects of a methyl donor-supplemented diet in female adult rats after experiencing a maternal separation paradigm during lactation.

2. Material and Methods

2.1. Animals

Timed-pregnant Wistar rats were provided on gestation day 16 from Charles River Laboratories (Portage, MI, USA). They were individually housed in a temperature (21±1°C)- and humidity (55±5%)-controlled room on a 12-h light/dark cycle, with food and water freely available. Every effort was made to minimize the number of animals used and their suffering. All the procedures were performed according to the European and national guidelines and were approved by the Animal Care Committee of the University of Navarra. Testing occurred during the light phase.

2.2. Maternal separation

All litters were born within a 2-day period and the number of litters used to generate the offspring tested in the experiments was 12. As previously described [30], at postnatal day (PND) 2, all pups were sexed and litters were randomly assigned to the control group (C, n=6), where pups were only briefly manipulated to change the bedding in their cages once weekly, or the maternal separation group (MS, n=6), where pups were daily separated from their dam for 180 minutes from PND 2 to 21 inclusive. Before manipulation of the MS pups, each dam was removed from its home cage and placed in an adjacent cage and, then, the pups were removed as complete litters, placed in an empty cage with standard bedding material, and transferred to an incubator in an adjacent room. To compensate for the mother's body heat, the temperature of the incubator was adjusted to the age of the neonates: 32 ± 0.5 °C (PND 2-5), 30 ± 0.5 ºC

(PND 6-14) or 28 ± 0.5 ºC (PND 15-21). After weaning on PND 23, rats were grouped in sets of eight and only females were chosen for the present study as a previous work of our group found that prenatal stress increased the obesogenic effects of a high-fat-sucrose diet only in offspring rats when adults, but only in females [31]. All subsequent experiments were performed when the rats were adult (60-75 days).

2.3. Experimental design and treatments

The dietary treatment commenced at 2 months because it is when the rats are considered adults according to previous works [32]. In this moment, C and MS animals were randomized by weight into two dietary groups and four different experimental groups were thus obtained. Two groups, non-stressed (C, n=8) and stressed (MS, n=8), were fed a standard chow diet (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Iberica, Barcelona, Spain), whereas two other groups, non-stressed (C+MD, n=8) and stressed (MS+MD, n=8), were fed the same standard diet supplemented with extra folic acid (5.55 mg/kg of diet) (Sigma-Aldrich, Saint Louis, Missouri, USA), vitamin B12 (0.551 mg/kg of diet) (Sigma-Aldrich), betaine (5 g/kg of diet) (Sigma-Aldrich), and choline (5.369 g/kg of diet) (Sigma-Aldrich) as described elsewhere (24). Animals had *ad libitum* access to water and food during the experimental trial (18 weeks) and body weight and food intake were recorded three times a week. The duration of the treatment (18 weeks) was chosen because DNA isolated from the brains of F344 rats fed a folate/methyl-deficient diet for 18 weeks showed a significant increase of 5-methyl-2-deoxycytidine content, in contrast with the livers of the same folate/methyl-deficient rats, which were characterized by profound loss of DNA methylation [33].

2.4. Behavioural testing

During the last three weeks of the 18 week-experimental trial three behavioural tests were carried out. To preclude that changes in locomotion might confound the behavioural data, horizontal locomotor activity, as total path length in cm, was measured in a softly illuminated room with a video tracking system (Ethovision 3.0, Noldus Information Technology, Wageningen, The Netherlands) for 30 min in an open field, which consisted of nine square arenas (43×45 cm^2, and height: 51 cm) made of black wood. The tracking system was set to determine the position of the animal five times per second.

To study depressive-like behaviour, the Porsolt forced swimming test was used as previously described [34]. Two swimming sessions were conducted: an initial 15-min pre-test followed 24 h later by a 5-min test. Rats were placed individually in a vertical plexiglass cylinder (height: 45 cm, diameter: 19 cm) filled with 28–30 cm of 26°C water. Immobility was considered as rats floating passively, making only small movements to keep its nose above the surface. Ethovision XT 5.0 (Noldus Information Technology) was used to score immobility times.

Novel object recognition (NORT) is a highly validated test for recognition memory. It was performed as described by Aisa *et al.* [35]. The open field consisted of a square open field (65cm*65cm*45cm) made of black wood. The previous day to the experiment, animals were familiarized with the field during 30 min. During the first trial of the experiment, two identical objects were placed within the chamber equidistant from the sides and the animal was allowed to freely explore for 5 min. It was considered that the animal was exploring the object when the head of the rat was oriented toward the object with its nose within 2 cm of the object. Sixty minutes later a second trial took place, in which one object was replaced by a different one, and exploration was scored

for 5 min. In order to eliminate olfactory stimuli, chamber and objects were cleaned after testing each animal. To avoid preference for one of the objects, the order of the objects was balanced between animals. Results were expressed as discrimination index: percentage of time spent with the novel object with respect to the total exploration time.

2.5. Tissues and blood collection

Animals were sacrificed by decapitation. Four rats from each experimental group were deeply anesthetized with 50-mg/kg intraperitoneal sodium pentobarbital. Animals were perfused (10 ml/min flow rate) intracardially with 0.1 M PBS (100 ml) and 4% paraformaldehyde in 0.1 M PBS (200 ml). Brains were removed, postfixed in the same fixative solution for 1 h at room temperature, and cryoprotected in 30% sucrose solution at 48°C. Freezing microtome sections of the dorsal hippocampus were cut coronally (30 μm) approximately at – 2.1 of bregma [36]. Sections were stored in 30% ethylene glycol, 30% glycerol, and 0.1 M PBS at -20°C until processed for immunohistochemistry. The brains of the other four rats from each experimental group were removed and dissected on ice to obtain the hippocampus and stored at -80 ºC for further analysis.

2.6. Serum measurements

Serum triglycerides (Randox LTD Laboratories, Ardmore Road, UK), glucose (ABX Pentra, Montpellier, France), total cholesterol (ABX Pentra, Montpellier, France) and HDL-cholesterol (ABX Pentra) and free fatty acids (FFA) (NEFA C kit, Wako Chemicals, Neuss, Germany) were measured in an automatized COBAS MIRA equipment (Rochel, Basel, Switzerland). Serum levels of homocysteine were analysed

(Homocysteine Enzymatic Assay; Demeditec Diagnostics, Kiel, Germany) using an automatized PENTRA C200 system (HORIBA Medical, Montpellier, France).
Plasma insulin levels were quantified by Elisa following the protocol described by the manufacturer (Linco Research, Missouri, USA). The quantitative insulin sensitivity check index (QUICKI) was calculated as the inverse of the sum of the logarithms of the fasting insulin and fasting glucose.
All assays were performed in duplicate. Inter-assay variability and intra-assay variability for insulin measurement were 8.5–9.4% and 1.4–4.6%, respectively. The sensitivity of the insulin assay was 0.15 mg/l.

2.7. DNA methylation

The immunohistochemistry procedure in the hippocampus was performed as previously described [37] with slight modifications. Mounted and dried sections were rehydrated and subjected to a sequence of incubation steps in a humidity chamber, starting with sodium citrate at 100 °C (pH 6) during 30 min for epitope recovery [38], followed by HCl 3.5 N during 15 min at room temperature with the purpose of exposing CpG islands, and finally with 3% hydrogen peroxide in methanol during 15 for blocking endogenous peroxidase. Sections were incubated overnight at 4 °C with 1:200 mouse monoclonal anti-methylcytosine antibody (Ref. 61479, Active Motif, Carlsbad, CA) followed by the appropriate streptavidin–biotin marked secondary antibody for 1h at room temperature (1:100, goat anti-mouse, Sigma-Aldrich) and VECTASTAIN®ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) for 1h at room temperature, consecutively. Peroxidase activity was visualized by incubating the sections with diaminobenzidine (DAKO Envision+ System-HRP, DAKO Corporation, Hamburg, Germany) and the sections were counterstained with Harris hematoxiline. All rinses

between the incubation steps were performed with TBST (Tris buffer saline–Tween, 0.05 M, pH 7.4). The negative control consisted of tissue sections treated without the primary antibody. The sections were viewed with a Nikon Eclipse E800 microscope equipped with a Nikon FDX-35 camera (Nikon Instech Co., Kanagawa, Japan). For the quantification of the area covered by the 5-methylcytosine positive staining, a series of hippocampal sections (4-5 sections per rat for each experimental group comprising 4 animals) were captured into images. The photographs were processed bilaterally and blindly with respect to MS and MD. All colour images obtained at the same magnification power were scaled and digitally transformed to 8-bit grey scale for analysis of DG layer by using the NIH ImageJ software version 1.43 (http://rsbweb.nih.gov/ij). The quantitative results are given in pixels, and they arise from 4 animals for each experimental group (C, MS, MD and MS+MD).

DNA methylation status of insulin receptor (IR) and corticotropin-releasing hormone (CRH) promoter regions were assessed in the hypothalamus. Genomic DNA was isolated with AllPrep® DNA/RNA kit following the manufacturer's instructions (Qiagen, Valencia, MD, USA). After bisulfite treatment (Epitect kit, Qiagen), the quantitative analysis of the methylation of 35 and 12 CpG sites (in IR and CRH gene promoters, respectively) was performed with MassARRAY Epityper (Sequenom, San Diego, CA, USA) by using the following primers:

CRH: Forward: TTTTTTTTGGTTTGTATTTGGTTTA;

CRH: Reverse: ACCTCCTACAAATTTTCTTCCTCTT;

IR: Forward: TTTTTGAAGTTGGGGGTTTTATAG;

IR: Reverse: CAAAATCCAAAACTCACCAAAATAC.

2.8. Western blotting

Hippocampal samples were homogenized in a 50 mM Tris buffer (pH 7.2, 4 °C). Each sample was adjusted to a final protein concentration of 4 mg/ml (DC protein assay; Bio-Rad, Hercules, CA). Extracts were mixed with Laemmeli's sample buffer boiled for 5 min. Samples (50 μg) were loaded onto 7.5 % bisacrylamide gels and separated by SDS-PAGE. Separated proteins were electrophoretically transferred from gels to PVDF membranes. IR protein was detected with anti-IR antibody (1:500; Cell Signalling Technology, Beverly, MA, USA). Immunopositive bands were visualized by a chemiluminescent method (ECL; Amersham, Arlington Heights, IL). The optical density of 135 kDa-reactive bands visible on X-ray film was determined densitometrically. β-actin was used as an internal control for normalizing IR expression.

2.9. Data analysis

All results are expressed as mean ± standard error of the mean (SEM). Data were evaluated by two-way ANOVA test (MD, MS, MD x MS) followed, if appropriate, by Tukey's least-significant difference post-hoc analysis. Repeated-measures ANOVA was applied for analysis of food intake and IPGTT results. The analyses of all the variables include the F-ratio of the interaction effects of the two factors, MS and MD and the probability of the interaction (p). The level of probability was set at $p<0.05$ as statistically significant. All analyses were performed using SPSS 15.0 package for Windows (Chicago, IL, USA).

3. Results

3.1. Body and biochemical measurements

At the end of the experiment, both the stress and the intake of a diet rich in methyl donors reduced the 18 week-body weight gain ($F_{(3, 31)} = 10.769$, $p<0.01$ and $F_{(3, 31)} = 8.415$, $p<0.01$ respectively) without affecting food intake (Table 1).

No changes were observed in circulating glucose and insulin levels at the end of the experiment, but MD and MS reduced total ($F_{(3, 31)} = 7.744$, $p=0.01$ and $F_{(3, 31)} = 8.301$, $p<0.01$, respectively) and HDL-cholesterol ($F_{(3, 31)} = 5.883$, $p<0.05$ and $F_{(3, 31)} = 10.910$, $p<0.01$, respectively) circulating levels (Table 1). Serum homocysteine levels did not vary in response to MS and MD, suggesting that this metabolite is not implicated in the effects observed in both conditions.

3.2. Behavioural testing

MS rats displayed a significant increase in the immobility time in the Porsolt forced swimming test, consistent with increased depression-like behaviour (Fig. 1). This effect was reversed by MD (Interaction (Diet x MS), $F_{(1, 26)} = 4.263$, $p<0.05$). These effects did not seem to be associated with changes in spontaneous locomotor activity, as the total distance travelled by animals was not modified either by MS or by MD (data not shown). As previously reported [34], MS induced a cognitive deficit as measured by NORT ($F_{(3, 31)} = 17.985$, $p<0.01$) that was not counteracted by MD. Discrimination indexes of C, MS, MD and MS+MD were 80.03±6.05, 52.06±7.62, 73.85±1.23 and 48.03±6.22, respectively.

3.3. Hippocampal expression of insulin receptor by Western blot

The two-way ANOVA analysis indicated a marginal trend towards significance in the interaction between MS and the diet ($F_{(1, 14)} = 4.191$, $p=0.068$). Although post-hoc analysis can not be performed when ANOVA does not reach the level of significance ($p<0.05$), this result suggests a possible decrease in IR levels in the hippocampus of MS rats compared to controls that was reversed by MD (Fig. 2).

3.4. 5-Methylcytosine immunohistochemistry

The immunoreactivity to 5-methylcytosine was assessed *in situ* in sections of hippocampus (Fig. 3a). Significant effects of MD were found in the dentate gyrus of hippocampus. Both groups that received methyl donors showed a significantly higher immunoreactivity ($F_{(1, 14)} = 10.819$, $p<0.05$; main effect of diet), indicating DNA hypermethylation as compared with the control group (Fig. 3b). There was not a statistically significant interaction between MS and MD supplementation.

On the other hand, no changes were observed in the DNA methylation status of IR and CRH promoter regions in the hypothalamus.

4. Discussion

In the current study we have evaluated the effects of the dietary MD during adulthood in rats exposed to MS during the lactation period. Exposure to stressors during the early stages of development has been shown to impact negatively on psychopathology in later life and has been associated with a range of mood and anxiety disorders [39]. For example, Benoit *et al*. [40] recently described that mice that underwent prenatal stress exhibited impaired spatial memory in the Morris water maze, that could be comparable with the deficit in the object recognition memory (as measured by NORT) that we have

found in the animals that suffered MS. Benoit *et al*. [40] suggested that these impairments could be related with epigenetic changes in the hippocampus, related with heterochromatin formation and reduced gene expression. Wei *et al*. [41] also found that mice exposed to brief daily separation for 3 hours, a model of early life stress, showed higher DNA methylation at the ribosomal DNA (rDNA) promoter, lower RNA levels and blunted hippocampal growth during the second week of life. The same model of 3-hour daily separation during the first 21 days of life has been previously associated with anxiety increase in adult rats [42]. And, among the genes whose expression was altered in a model of prenatal stress in mice, both in the frontal cortex and hippocampus, it was *DNMT1*, that was accompanied by an enrichment in 5-methylcytosine and 5-hydroxymethylcytosine at *BDNF* gene regulatory regions and a decrease in *BDNF* expression [43]. However, in the current experiment we have not found differences in the levels of 5-methylcytosine immunostaining in hippocampus between MS and control animals. Our group has previously reported a significant decrease in hippocampal *BDNF* expression in the same rat model of 3-hour daily maternal separation [44]. A recent study has reported that methyl supplementation via methionine is able to increase *BDNF* DNA methylation and reduce *BDNF* mRNA levels in the hippocampus of epileptic rats during memory consolidation, reversing memory deficits in these animals [45]. This result suggests that methyl donor supplementation as performed in our study (extra folic acid, vitamin B12, betaine and choline) could also be able to reverse the detrimental effects of early life stress on *BDNF* methylation and expression in rat hippocampus, although more studies are required.

In agreement with our results, it has experimentally been demonstrated that prolonged episodes of MS during the first weeks of life induced depression-like behaviour in adult rats [5,35], including studies [46]. The main finding of this research is that dietary

supplementation with methyl donors in adulthood reversed the immobility time in the Porsolt forced swimming test, ameliorating the depression-like phenotype induced by MS.

SAM is the main methyl donor for the various methyltransferase enzymes, and the nutrients that play a role in its metabolism have been described to influence various cognitive processes, in part affecting DNA methylation [47]. Thus, folic acid, vitamin B12, and other nutrients influence the function of enzymes that participate in various methylation processes by affecting the supply of methyl groups that these enzymes incorporate into a wide variety of molecules [48]. In our study, MD decreased body weight gain and increased total and HDL-cholesterol. In other animal model (*agouti* mice), Waterland *et al.* [49] described that maternal MD prevented transgenerational increase in adult body weight. Previous investigations had already described the increase of HDL-cholesterol due to betaine [50] and folic acid supplementation [51] in mice and humans, respectively. However, early-life stressed animals gained less weight and had lower total and HDL-cholesterol levels than the control rats. The results of the lipid profile are difficult to interpret because the Wistar rat is not a good model of dyslipidemia, and we must be very cautious about attributing a similar effect of MD in humans. However, this and previous studies [50, 51] suggest that methyl donor supplementation could be a promising strategy to increase HDL-cholesterol levels and normalize the lipid profile, which merits further studies in in more specific animal models of dyslipidemia and atherosclerosis, such as Golden Syrian hamster fed a high-fat, high-cholesterol, and high-fructose, low-density lipoprotein receptor (*LDLR*) knockout mice or apolipoprotein E-deficient ($Apoe^{-/-}$) mice fed a high-fat diet. In this sense, it has been reported that maternal diet supplemented with methyl-donors protects against atherosclerosis and ameliorates atherosclerosis development in F1 Apoe−/−

mice [52], which has been attributed to the inhibition of the T-cell CCR2 expression and the reduction of inflammatory cytokine production

Regarding glycemic control, fasting variables were not modified, suggesting that not peripheral, but central insulin signalling could be affected. In this context, a trend toward decrease in the expression of IR in the hippocampus due to MS was partially (although not statistically significantly) reversed by MD. It is well known that maternal stress alters endocrine function of the fetus, including IR expression in the brain. According to Grillo et al. [53], hippocampal insulin resistance may be a key mediator of cognitive deficits, independent of glycemic control. These authors observed that hippocampal neuroplasticity was impaired in a model of hippocampal-specific insulin resistance, although no effects on body weight, adiposity and glucose homeostasis were found. This result is in accordance with our data, in which IR expression tended to be downregulated in the hippocampus of the MS group and was accompanied by an increased depression-like behavior (Porsolt test) and a cognitive deficit (as measured by NORT). The fact that MD supplementation tended to reverse immobility time in the Porsolt test (although not the NORT results) suggests that this beneficial effect could be related to the trend towards significance in the interaction between MS and MD in the hippocampal expression of IR. Previous studies have reported that neonatal overnutrition could regulate IR in the brain (hypothalamus) via DNA methylation of its promoter [54]. However, in our study, no differences in IR methylation in the hypothalamus were observed as a result of MS and MD. Moreover, MS paradigm did not change the weaning body weight of or animals, suggesting that differences in early life nutrition do not seem to be behind our results.

As described in the introduction, different environmental factors could modify the epigenetic marks in different regions of the brain. In the current study, an increase in

total DNA methylation levels was observed in hippocampus due to the intake of a diet rich in methyl donors. Moreover, IR expression in the hippocampus was decreased by MS and reversed by MD. In this sense, studies in rodents have previously focused on the protective effects of dietary supplementation with methyl donors on obesity [49] and hepatic fat accumulation [29,55], suggesting an improvement of diet-induced insulin resistance by activating IR and the downstream signalling pathways.

The promising effects of MD in the reversion of early life stress-induced neurological alterations suggest the design of new experiments. Among the different points that merit further study, there are: 1) to test if the beneficial effect is due to the mix of methyl donors or there is one or two of them that are more powerful; 2) to administer the MD cocktail to other models of chronic mild stress in adults; 3) to study whether the effect of MD could be more beneficial if it is supplemented to the mothers during weaning; 4) to analyze the effect of MD on brain and peripheral inflammatory biomarkers associated with metabolic and neurological diseases.

5. Conclusion

Maternal separation (180 minutes from postnatal day 2 to 21) reduced body weight and total and HDL-cholesterol levels without affecting food intake. It also induced a depressive-like behaviour in the Porsolt test and downregulated IR expression in the hippocampus. In control rats, MD affected neither circulating glucose and insulin levels nor Porsolt results, but induced DNA hypermethylation in the hippocampus, as shown by immunohistochemistry. Finally, MD reversed the deleterious effect of maternal separation on total and HDL-cholesterol levels and the Porsolt test. Some of these effects could be mediated by the methylation of specific CpG sites in the DNA of key regulatory genes in the brain.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to Veronica Ciaurriz for her technical laboratory support. We would like to thank "*Asociación de Amigos de la Universidad de Navarra*" and IBERCAJA (Spain) for the doctoral fellowships of Laura Paternain and Eva Martisova. The authors wish to thank Línea Especial (LE/97) from the University of Navarra and the Carlos III Health Institute (CIBERobn) for financial support.

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Figure legends

Fig. 1 Effects of methyl donor supplementation (MD) on maternal separation-induced depressive-like behaviour in the Porsolt forced swimming test. Results are expressed as mean ± SEM and were analysed with two-way ANOVA (Diet x MS). Interaction (Diet x MS) ($F_{(1, 26)}$= 4.263, $p<0.05$). $^{\dagger}p < 0.05$ vs. MS, $^{\$}p<0.05$ vs. C. C: control, MS: maternal separation.

Fig. 2 Effects of maternal separation or methyl donor supplementation (MD) on the expression of insulin receptor (IR) in hippocampus. Results are expressed as mean ± SEM of the percentage of optical density (O.D.) of controls and were analysed with two-way ANOVA (Diet x MS). Interaction (Diet x MS) ($F_{(1, 14)}$ = 4.191, $p=0.068$). $^{\$}p<0.05$ vs. C, $^{\dagger}p<0.05$ vs. MS. C: control, MS: maternal separation.

Fig. 3 Immunoreactivity to 5-methylcytosine after maternal separation or methyl donor supplementation (MD) in hippocampus. (A) Photomicrographs of 30 µm coronal sections showing 5-methylcytosine positive cell nuclei (arrows) in dentate gyrus of hippocampus. Scale bar 100 µM. (B) Graphic representation of immunoreactivity to 5-methylcytosine. Results are expressed as mean ± SEM and were analysed with two-way ANOVA (Diet x MS). $^{*}p<0.05$, main effect of diet. C: control, MS: maternal separation.