Sexual dimorphism modulates metabolic and cognitive alterations under HFD nutrition and chronic stress exposure in mice. Correlation between spatial memory impairment and BDNF mRNA expression in hippocampus and spleen

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Abbreviations: HFD: High-Fat Diet, CD: control diet, CMS: Chronic Mild Stress, VF: visceral fat, SF: subcutaneous fat, 2hPG: blood glucose levels at 120 min after glucose injection, HOMA IR: Homeostasis model assessment of insulin resistance, TG: triglycerides, BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor, NT3: neurotrophin 3, IL-1 beta: interleukin one beta, IFN-gamma: interferon gamma.

ABSTRACT

Aims: The accumulated evidence suggests that lifestyle - specifically dietary habits and stress exposure - plays a detrimental role in health. The purpose of the present study was to analyze the interplay of stress, diet, and sex in metabolic and cognitive alterations.

Main methods: For this purpose, one-month-old C57Bl/6J mice were fed with a standard diet or high-fat diet (HFD). After eight weeks, one subgroup of mice from each respective diet was exposed to 20 weeks of chronic mild stress (CMS), whilst the others were left undisturbed.

Key findings: After 28 weeks of HFD feeding, mice from both sexes were overweight, with an increase in caloric intake and abdominal and subcutaneous fat pads. Stress exposure induced a decrease in body weight, related to a decrease in caloric efficiency in both males and females. Results indicate that males are more susceptible than the females in modulating metabolic and cognitive functions under HFD and CMS. Although both sexes demonstrated HFD-induced weight gain, fat accumulation, insulin resistance, high cholesterol, only males exposed to CMS but not females have (i) impaired glucose tolerance with higher glucose level; (ii) significant prolonged latency in Barnes test, suggesting cognitive impairment; (iii) increased IFN-gamma expression in hippocampus, suggesting greater neuroinflammatory response; (iv) poorer cognitive performance related to a decrease in hippocampal and spleen BDNF mRNA expression.

Significance: The main finding in this study is the presence of a sexual dimorphism in modulating metabolic and cognitive functions under HFD and CMS, showing males are more susceptible than females. In addition, poorer cognitive performance was related to a decrease in hippocampal BDNF mRNA expression. Interestingly, these changes were observed in the spleen as well.

Key words: High-fat diet, chronic stress, metabolism, cognition, neurotrophins, peripheral lymphocytes.

1. Introduction

Lifestyle - specifically dietary habits and stress exposure - plays a detrimental role in health. Overweight and obesity are defined as aberrant or excessive fat accumulation. According to a report broadcasted by the World Health Organization in 2018, the incidence of obesity has nearly tripled since 1975 (World Health Organization). Obesity-related metabolic disorders not only affect systemic health but also brain function. It was found that obesity may result in neurodegeneration, cognitive impairment, and increased susceptibility to brain damage (Ashrafian et al., 2013; Zanini et al., 2017; Tsan et al., 2021).

In the context of obesity-induced cognitive decline, the neuroinflammation generated in the hippocampus -a limbic area involved in learning and memory - that has been associated with changes in the integrity of the blood-brain barrier (Van Dyken and Lacoste, 2018) is of particular interest. Cytokines such as IL-1 beta, IL-6, IFN-gamma, TNF-alpha, MCP1, have been involved in the generation of neuroinflammation and the increase in oxidative stress, leading to cognitive decline (Castanon et al., 2015).

Habitual consumption of calorically dense foods during early life developmental periods leads to a prevalence of childhood obesity. The World Health Organization reports that the prevalence of overweight and obesity among children and adolescents aged 5-19 has risen dramatically from just 4% in 1975 to just over 18% in 2016, with over 340 million children and adolescents being overweight or obese (World Health Organization). These eating habits acquired in childhood are strongly associated with a higher chance of obesity in adulthood (Reilly and Kelly, 2011; Tsekoura et al., 2021).

Likewise, the normal structure and function of the brain are altered by long-term stress (McEwen, 2017). Specifically, the hippocampus is particularly vulnerable to the effects of stress (McEwen, 2017; Palumbo et al., 2012; Yoshioka et al., 2022). It was

suggested that repeated exposure to stress confers a higher risk of developing neurodegenerative diseases (Mohammadi et al., 2021; Bisht et al., 2018; Donley et al., 2018).

A relation between chronic stress and eating behavior has been described in humans (Debeuf et al., 2018; Pickett et al., 2020). Similarly, several animal models showed an association between chronic stress and obesity with divergent metabolic phenotypes (Patterson and Abizaid, 2013; Balsevich et al., 2019). It was found that chronic stress raises the consumption of palatable foods resulting in obesity (Bartolomucci et al., 2009; Wei et al., 2019). However, other studies reported that the exposure to daily stress does not facilitate or exacerbate obesity even in obesity-prone laboratory rodents (Michel et al. 2005).

It has been reported that sex differences in cognition after chronic stress exposure strongly depend on the strain used (Franceschelli et al., 2014; Hodes and Epperson, 2019). Similarly, the role of sex and sexual hormones on metabolic diseases has been thoroughly described (Bale and Epperson, 2017; Varghese et al., 2021). The relevance of the inclusion of sex as a biological variable in research to improve the understanding of disease mechanisms was heavily emphasized (Mauvais-Jarvis, 2017; NIH, 2021). However, despite evidence supporting a link between diet and stress, as well as memory impairment and metabolic changes, few studies investigate the interplay of these factors on the metabolic and cognitive outcomes in both females and males.

Neurotrophins are a family of secreted proteins that control neuron survival and development, and regulate neuronal plasticity (Skaper, 2018). The members of this family are brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin NT4/5 (Al-Qudah and Al-Dwairi, 2016). Particularly, BDNF has been implicated in learning and memory formation (Bekinschtein et al., 2007).

Interestingly, it has been proposed that serum levels of BDNF could be correlated to cognitive function, but conflicting data were found in patients with Alzheimer's disease and individuals with mild cognitive impairment (Ng et al., 2019). Additionally, it was found that BDNF mRNA expression in leukocytes is related to Frontal Assessment Battery scores in crack-cocaine and alcohol use disorder patients (Anders et al., 2020).

Hence, the aim of this study is to evaluate if chronic stress exposure aggravates the metabolic changes induced by a high-fat diet (HFD), if obese mice are vulnerable to stress-induced cognitive deficit in male and female mice, and whether these effects are related to changes in neurotrophin and cytokine levels in the hippocampus. Moreover, we analyzed if the molecular changes in the hippocampus are also found in the spleen to propose lymphocytes as peripheral marker of susceptibility to behavioral alterations induced by HFD and/or stress exposure. For this purpose, HFD feeding began at one month of life and stress exposure at 3 months of age to investigate the effects of an obesogenic diet during infancy and adolescence on metabolism and cognitive behavior outcomes following stressors exposure during adulthood

2. *Materials and Methods*

2.1. Animals and diet

Male and female C57Bl/6J mice were born and housed in the animal's facility at the Instituto de Investigaciones Biomédicas (BIOMED-CONICET-UCA, Argentina). Four mice were located by cage and maintained with 12 hours' light-dark cycles, beginning at 7:00 AM and 7:00 PM respectively and kept under controlled temperature (20 ± 2 °C). Unless stated otherwise, food and water were freely available. Animals were taken care of and euthanized according to norms of the "Guide for the Care and Use of Laboratory Animals"

(NIH) (revision 2011) and to the EC Directive 86/609/EEC (revision 2010). The experimental protocol was approved by the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina) under resolution 2947/13 and endorsed by (CICUAL, BIOMED-CONICET-UCA, Argentina) under resolution 0002/2018. In addition, the authors had performed the checklist recommendations according to ARRIVE guidelines.

Two types of diets were used in this study, standard chow diet (ACA nutrition animal Cat n° 16-014007) named control diet (CD, 2.96 kilocalorie/g) and HFD (4.37 kilocalorie/g). The most frequent model for studying obesity is the diet-induced obesity which provides highly human-like models. There are numerous obesity-inducing diets, including high-fat diets and cafeteria diets. In HFDs, various fat sources are used in different proportions (Bortolin et al., 2018). We choose a high-fat diet as similar as possible to the human diet that is a diet with high-fat (lard and butter). HFD was prepared by mixing standard chow diet with dairy butter and porcine lard in a 1000:225:130 proportion with the addition of a vitamin B complex. To avoid spoiling, HFD was replenished twice a week. The macronutrient, vitamin and mineral requirements of the two diets are in accordance with the recommended composition and concentrations for adult maintenance in the AIN-93M Purified Diets for Laboratory Rodents (Reeves et al., 1993). The macronutrient composition of each diet is presented in the table insert in Fig. 1.

2.2. Chronic Mild Stress Model (CMS).

The CMS model was conducted as previously described (Palumbo et al., 2018). Animals were housed individually and CMS consisted of randomized stressors alternately applied one a day throughout the week. These were: two periods of continuous overnight illumination, 8 h overnight water deprivation, one period of food deprivation (12 hours: 6 h

during the light and 6 h during the dark period), two periods (7 and 17 h) of 45°C cage tilt, and one 24-h period of paired housing (mice were always housed in the same pairs, but the location alternated between the home cages of each member of the pair). All the individual stressors used have been filed as “mild” according to the Animals (Scientific Procedures) Act of 1986 (UK legislation).

2.3. Experimental design

Figure 1 show the experimental design used in the present work. A total of 64 males and 64 females four-week-old C57Bl/6J mice were randomly assigned to one of two groups: one group, namely CD , were fed *ad libitum* with standard chow diet (2.96 kcal/g), the other group, namely HFD, were fed with a HFD (4.37 kcal/g) for 28 weeks to induce metabolic alterations (Reverte et al., 2020). After eight weeks, one subgroup of mice of each diet was randomly allocated to 20 weeks of CMS exposure or left undisturbed. The combination of these conditions resulted in the following four groups: 1) CD + undisturbed group (CD), 2) CD + stress group (CMS), 3) HFD + undisturbed group (HFD), and 4) HFD + stress group (HFD+CMS). Animals were utilized for behavioral testing and biochemical and molecular determinations. After behavioral testing, mice were left undisturbed in their home cages for 48 h before sacrifice. The 6 hours fasted animals were anesthetized with CO_2 over-exposure and blood was drawn from the retro-orbital venous sinus and then the animals were sacrificed by cervical dislocation. Whole blood was collected in a tube containing 30 ul 0,1M EDTA and plasma was separated in a refrigerated (4ºC) centrifuge. Visceral (abdominal) and subcutaneous (inguinal) fat pads were removed. Hippocampus and spleen were dissected immediately on ice and stored at −80 °C until use. Hippocampus was extracted according to Mouse Brain Anatomy Atlas (Paxinos and Franklin, 2008).

2.4. Body weight and food intake evaluation

Body weight and food intake were measured twice a week during the treatment. The caloric intake was calculated for each home cage by multiplying the food intake (in grams) by the Calories of each diet expressed as kcal/g. After sacrifice, abdominal (visceral fat, VF) and inguinal (subcutaneous fat, SF) fat were removed and weighed. Results are expressed as the percentage of each adipose tissue weight relative to animal body weight.

2.5. Blood glucose determinations

Blood glucose measurements were performed through a small skin incision at the tip of the tail. Glycemia was determined at basal conditions, after 6 h of fasting (from 8:00 AM to 2:00 PM) with free access to water. Immediately after, animals were intraperitoneally injected with 2 g/kg body weight of D-glucose (Sigma Cat N° G7021) and glycaemia was measured at 120 min after injection (2hPG) (Inzaugarat et al., 2017). Since in HFD-mice higher body weight is due to an increase in fat mass, but the mice do not have a higher lean mass – the principal site of glucose disposal – the glucose mass injected was the same for all mice, regardless of treatment. Otherwise, obese mice could be misdiagnosed as being glucose intolerant simply because they receive more glucose for the same lean body mass (McGuinness et al., 2009). All the blood glucose samples were measured using reactive strips and a reading apparatus (Lifescan Inc, Great Britain; useful range, 20–600 mg/dl).

2.6. Serum insulin measurement

Serum insulin concentration was determined by a radioimmunoassay (RIA) using humanized porcine insulin for iodination and standard, provided by Laboratorios Beta

(Buenos Aires, Argentina), and anti-bovine/ human/ pig insulin antibody (Sigma Cat N° I8510) (Bianchi et al., 2021; García-Tornadú et al., 2010). Minimum detectable concentrations were 0.08 ng and intra- and inter-assay coefficients of variation were 7.2 ± 1.1 and 11.4 ± 1.5 % respectively.

2.7. Homeostasis model assessment for insulin resistance

To evaluate the degree of insulin resistance, the homeostasis model assessment (HOMA) was calculated using the following formula: HOMA IR = fasting serum insulin (μU/ml) x fasting blood glucose (mmol/l)/22.5 (Matthews et al., 1985).

2.8. Lipid profile

Triglycerides were determined in plasma by a standardized method using TG Color Reagent (Wiener Lab, Rosario, Argentina Cat N° 1780111) and total cholesterol and HDL cholesterol were quantified using Colestat enzimático Reagent (Wiener Lab, Rosario, Argentina Cat N° 1220110 and 1361402 respectively). Values were normalized to the control group of each plate.

2.9. Behavioral tests

Behavioral tests were conducted between 5:00 PM and 7:00 PM and recorded using a video camera (Sony DCB-DVD810), as previously reported (Pascuan et al., 2017; Palumbo et al., 2018). Data were obtained by manual observation of the videos. To avoid any influence of the experimenters, one person recorded the video and the other one watched them without knowing which group do animals belong to. All animals were used for spontaneous alternation and habituation tests (week 26th). After a week (week 27th) a

group of animals randomly selected were trained and then tested in Barnes Maze. Since this test involves learning these animals were not used for neurotrophin analysis.

2.9.1. Open-field habituation

To assess contextual non-associative learning and memory an open-field test was performed (Walsh and Cummins, 1976). For this purpose, the animals were placed for 5 min into the center of a rectangular chamber (l × w × h, 42 × 35 × 15 cm) divided into 30 squares of 7 × 7 cm. The behavioral parameters that were determined included crossed lines (horizontal activity), and the number of times a mouse stood on its hind legs (rearing, vertical activity). After 24 h, the mice were re-exposed to the arena and the same parameters were measured again. The habituation was defined as a significant decrease in exploratory activities between both days.

2.9.2. Y-maze spontaneous alternation

To evaluate spatial working memory we determined the spontaneous alternation in a Y-maze with spatial clues (Martínez-Díaz et al., 2015). The Y-maze apparatus consisted of three identical black plexiglass arms (30 cm long) joined by a common central platform. At the beginning of the session, the mice were put into the central platform and arm entries were recorded for 5 min. An alternation was described as three successive entries into each arm of the maze without any repeated entry. The percent of alternation was calculated according to the following formula: [number of alternations / (total arm entries-2)] x 100.

2.9.3. Barnes Maze

The Barnes maze test evaluates spatial learning and memory and it is based on the natural tendency of mice to avoid open spaces and bright lights and preference of dark confined spaces (Dawood et al., 2004). The apparatus is a grey-coated opaque circular surface 90 cm in diameter with sixteen 5 cm wide holes located around the platform, elevated 75 cm above the floor and located under bright overhead lighting. The escape box (17 cm×13 cm×7 cm) was a grey store box with a 5 cm diameter hole in the lid and it represented the target. Visual cues are placed around the apparatus. The protocol performed here was carried out in stages over 5 days, and included a habituation phase (Day 1), acquisition phase (days 2-4) and probe testing (day 5). Habituation and acquisition periods consisted of four daily trials. In initial trials, the scientist gently leads the animal to the drop box. In subsequent trials, the animal is placed in the center of the table and must find the drop box on its own. After a few trials, rodents typically remember which hole contains the drop box and quickly proceed in a direct path toward the hole. The escape box was always located underneath the same hole (stable within the spatial environment), which was randomly determined for each mouse. In between each trial, the maze was cleaned with 70% ethanol to remove odor cues. For the probe trial, the escape cage was removed and the following parameters were measured: the amount of time the mice spent exploring in the target quadrant (where the target hole is located) and non-target quadrants and the latency to locate the target hole. Exploration is defined as nose pokes and/or head deflections into any hole, and errors are those realized in non-target holes.

2.10. Quantification of mRNA Expression

qRT-PCR was performed as previously described (Palumbo et al., 2018; Pascuan et al., 2017). Briefly, total RNA was isolated from hippocampi and spleen by homogenization in Tri-Reagent (Molecular Research Center Cat N° TR 118) according to the manufacturer's instructions. RNA was converted to cDNA using oligo (dT) primers and M-MLV reverse transcriptase with deficient RNase H activity (Easy Script reverse transcriptase [M-MLV, RNase H-] Transgenbiotech Cat N° TGB-AE10102-G). Real-time PCR was performed for quantitative assessment of mRNA expression with an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the KAPA SYBR®FAST qPCR Kit Master Mix (2×) Universal (Kapa Biosystems Cat N° KK4602), run in duplicate. The sequences of mouse-specific primers, annealing temperature, and amplicon size are provided in Table I. To determine the target gene mRNA expression, the comparative cycle threshold (Ct) method was used (Livak and Schmittgen, 2001. An average Ct value was calculated from the duplicate reactions and normalized to the expression of the reference gene (GAPDH). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found to be the most stable reference gene for testing expression levels among other housekeeping genes tested (β-actin and β2-microglobulin) before starting the experiment.

2.11. Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) for each group. All the data were processed using STATISTICA (StatSoft, Inc., Tulsa, OK, USA), INFOSTAT software (Universidad Nacional de Córdoba, Argentina) and IBM SPSS statistics. The normality and homogeneity of variance for the dataset were tested using the Shapiro–Wilk and Levene's test. When these assumptions were not met, the data were

transformed appropriately. Parameters that involved more than one measurement over time with the same subject were evaluated with the General Linear Model (GLM) Repeated Measures (RM) Factorial ANOVA with sex (female and male), diet (control or HFD), and stress exposure (CMS exposure or undisturbed) and time as factors. When more than three repetitions were analyzed, sphericity test was performed and if sphericity is violated, Greenhouse-Geisser adjustment was used. Planned comparisons were performed to determine the significance level. When time was not a factor, data were analyzed using GLM Factorial ANOVA. If a significant interaction was obtained, simple effects analysis was performed. In all cases, Fisher's Least Significant Difference (LSD) post-hoc test was applied to compare the data between groups. For those variables that did not yet meet the ANOVA assumptions when transformed, Kruskal-Wallis non-parametric test was performed. $p<0.05$ was considered to indicate a statistically significant difference. To analyze correlations between cognition and BNDF levels Spearman correlation was used for data normally distributed and Pearson correlation test for non-parametric distribution.

3. Results

3.1. HFD-feeding increased body weight and fat content associated to caloric intake in males and females. Chronic stress exposure resulted in a decrease in body weight not related to caloric intake.

To verify if HFD induces overweight, we determined the body weight across the weeks and the fat accumulation at the end of the experiment. In addition, as it was reported a relationship between chronic stress and eating behavior, we studied the effect of CMS exposure on both parameters. As can be seen in figure 2 A and B, males and

females increased their body weight throughout the experiment. RM ANOVA indicated that body weight curve depending of diet, sex and CMS exposure (interaction time*diet*sex*CMS ($F_{(12.84, 1540.43)}$= 3.128 (corrected by Épsilon of Mauchly's sphericity test) p<0.0001). Using planned comparisons, we observed that male and female mice fed with HFD developed an increase in body weight greater than those fed with CD. CMS exposure decreased body weight independently of diet for males and females (Fig. 2 A and B).

At the 28th week of diet, 20th week of stress exposure, the factorial ANOVA test indicated a significant effect of diet ($F_{(1,120)}$= 107.04, p<0.0001), stress ($F_{(1,120)}$= 41.14, p<0.0001) and sex ($F_{(1,120)}$= 293.13, p<0.0001) without interaction between them. Post-hoc analysis revealed a higher body weight in males fed with HFD respect to those fed with CD at the 28th week (body weight (g), mean ± SEM, HFD= 37.0 ± 0.6 vs CD= 33.0± 0.5, p<0.0001). Similar results were found in females (body weight (g), HFD= 30.2 ± 1.0 vs CD=24.8 ± 0.3, p<0.0001). CMS exposure decreased body weight gain in both males and females fed with CD or HFD. In fact, a significantly lower weight was observed in males fed with CD (body weight (g) CD+CMS= 30.4 ± 0.5 vs CD= 33.0 ± 0.5, p=0.0144) and in animals under HFD (body weight (g), HFD+CMS= 34.5 ± 0.6 vs HFD= 37.0 ± 0.6, p=0.0075). Similar decrease was observed in females fed with CD (body weight (g), CD+CMS= 23.9 ± 0.1 vs CD=24.8 ± 0.3, p=0.0033) and in those fed with HFD (body weight (g), HFD+CMS = 25.7 ± 0.4 vs HFD= 30.2 ± 1.0, p<0.0001).

The increase in body weight was in accordance with a higher fat pad mass (Fig. 2 C and D). Kruskal-Wallis analysis (H (7) = 100.68 p<0.0001) indicated a significant increase in VF in animals of both sexes fed with HFD. Stress exposure did not induce any changes in males fed with CD or HDF. However, stress induced a significant reduction in female fed with HFD (Fig. 2C).

For subcutaneous fat (SF), Kruskal-Wallis analysis (H (7)=109.96, *p*<0,0001) indicated a significant increase of SF in HFD-mice compared to CD in both males and females. Stress induced a non-significant decrease of SF in both male and females feed with HDF, indicating that those with CMS were more resistant to weight gain and subcutaneous fat accumulation (Fig. 2D).

Considering that the amount of Calories differed between CD and HFD, the intake was estimated as individual caloric intake per week (Fig. 2 E and F). RM ANOVA indicated that intake was depend on sex ($F_{(1,24)}$= 7.23 p=0.0130) and interaction diet x time ($F_{(2.431, 58.35)}$ = 4.90, corrected by Épsilon of Mauchly's sphericity test, p=0.007), but not stress ($F_{(1,24)=}$ 0.05, NS). Planned comparison showed significant differences between diets along the time (*p*<0.0001). As can be seen in Fig 2 E and F the caloric intake was significantly higher for HFD mice compared to CD in both males and females, without an effect of CMS for both diet and sex.

3.2. High-fat diet and/or stress altered glucose and lipid metabolism with a worse profile in males than in females

Overweight and stress are frequently associated with metabolic changes, so we studied the effect of HFD and/or stress exposure on glucose and lipid metabolism.

Concerning glucose metabolism after 28 weeks of feeding with CD or HFD and 20 weeks of stress exposure, RM-ANOVA of glucose levels, both basal and after 2 hours glucose administration (2hPG), indicated an interaction of time x sex ($F_{(1,120)}$ = 17.66, *p*=0.0001), time x diet ($F_{(1,120)}$ = 7.97, *p*= 0.0056) and time x CMS $F_{(1,120)}$ = 4.13, *p*< 0.0443, sex x diet $F_{(1,120)}$ = 17.65, *p*< 0.0001) sex x CMS $F_{(1,120)}$ = 4.35, *p*< 0.039 . As can be seen in figure 3A, after 2 hours of glucose administration, males fed with CD recovered basal values. However, males subjected to stress, HFD or both, kept high blood glucose values

which were significantly different from basal values. In contrast, females recovered basal values independently of the diet or stress exposure (Fig. 3A). It is interesting that at 2h glucose level detected in HFD+CSM seemed to be much higher than HFD or CMS alone in males. To confirm this appreciation, we calculated the fold change of glucose levels at 2h respect to fasting levels (insert Fig. 3A). Factorial ANOVA showed that the fold change was dependent of sex ($F_{(1,120)}$ =14,60, p= 0.0002) and diet ($F_{(1,120)}$ = 8.80, p< 0.0036). Post-hoc analysis indicated that males fed with HFD and exposed to CMS have a significant increment respect to CD and HFD alone.

Respect to insulin values, factorial ANOVA indicated that there was an effect of diet ($F_{(1,96)}$= 23.74, p=0.0001) and sex ($F_{(1,96)}$= 99.61, p<0,0001) but not of stress ($F_{(1,96)}$= 0.17, NS). As can be seen in figure 3B, post-hoc analysis did not shown differences in serum insulin values for any experimental condition for males, but an increase was observed for females fed with a high-fat diet independent of CMS treatment.

HOMA-IR, a measurement of insulin resistance, was dependent on diet ($F_{(1,96)}$=30,70, p=0.0001) and sex ($F_{(1,96)}$=97.10, p<0,0001) but not stress exposure ($F_{(1,96)}$=0.25, NS). For males, HOMA-IR was modified by HFD, independently of stress exposure. Similar results were found for female (Fig. 3C).

Regarding the cholesterol levels factorial ANOVA showed an interaction between diet, sex and stress ($F_{(1,120)}$=5.28, p=0.0233). Simple effect analysis indicated an increase of total cholesterol in males fed with HFD, without or under stress ($F_{(1,120)}$=48.90, p<0.0001) (Fig. 3D). For females, there was an increment for mice fed with HFD ($F_{(1,120)}$=11,10 p=0.0110) and a differential effect of CMS, a significant decrease for CD (($F_{(1,120)}$=4.44 p=0.0371) and a significant increase for HFD ($F_{(1,120)}$=6.67, p=0.0110).

For HDL cholesterol Kruskal-Wallis analysis (H (7)= 86.75 p<0,0001) indicated a significant effect of HFD, showing a similar profile to total cholesterol (Fig. 3E).

Respect to TG levels, Kruskal-Wallis analysis (H (7) = 19,96 p<0,0057) indicated a significant effect between factors. However, post hoc analysis did not identify differences between groups, as can be seen in figure 3F.

3.3. High-fat diet feeding and chronic stress exposure impaired cognitive performance in males.

As mentioned above, an association between overweight and impaired cognitive function was described. In addition, it was reported that chronic stress exposure affects learning and memory. Taking into account these findings we evaluated cognitive performance in mice fed with HDF and/or exposed to CMS. For this purpose, habituation in an open field, spontaneous alternation and spatial learning and memory tests were performed.

Figure 4 A and B shows open field results. As expected, RM ANOVA indicated a significant effect of time with a significant decrease in both horizontal ($F_{(1,120)}$=321.77, p<0.0001) and vertical ($F_{(1,120)}$=125.27, p<0.0001) exploration activity 24 h post-training for males and females, fed with CD or HFD and non-exposed or exposed to CMS. Figure 4C displays the spontaneous alternation in the Y-maze task. Factorial ANOVA indicated an interaction between sex, diet and stress ($F_{(1,120)}$=4.60, p=0.0340). Simple effect analysis showed that CMS had an effect on male under CD ($F_{(1,120)}$=12.10, p=0.0007)) but not for those fed with HFD ($F_{(1,120)}$=0.93, NS). Post-hoc analysis revealed that both stress and HFD significantly decreased the spontaneous alternation in males, but not in females.

In respect to the Barnes maze task, results showed that the percentage of time spent in the target quadrant was similar for all experimental groups (between 50-60 %, figure 4E). But the latency to find the target hole had an interaction between sex, diet and CMS

($F_{(1,56)}$= 4,58, p= 0.0500). Subsequent analysis showed a significant effect of CMS in males with HFD ($F_{(1,56)}$= 7.33, p= 0.0090) (Fig 4D).

3.4. *High-fat diet feeding and chronic stress exposure induce a reduction in brain-derived neurotrophic factor mRNA expression and an increase of IFN-gamma in hippocampus*.

To analyze if the cognitive deficit was related to changes in neurotrophin levels in the hippocampus, mRNA expression of BDNF, NT-3 and NGF was determined. Kruskal-Wallis analysis did not showed any significant differences between groups for NGF mRNA levels (KW *H* (7) =4.75, NS). Similar results were observed for mRNA levels of NT3 (KW *H* (7) =7.58, NS) (figure 5 B and C). However, factorial ANOVA showed an interaction between diet and sex in BDNF ($F_{(1,56)}$= 17.48, p=0.0001). Analysis of simple effects indicated that there is an effect of diet in males ($F_{(1,56)}$= 21.61, p<0.0001) but not in females ($F_{(1,56)}$= 1.23, NS). Post-hoc test showed that males fed with HFD had a significant decrease of BDNF. However, exposure to CMS did not induce a significant effect in CD or HFD mice (Fig. 5 A).

It was described that mice fed with a HFD presented neuroinflammation related to blood-brain barrier disruption that in turn induce behavioral alterations (Salas-Venegas et al., 2022). Taking into account these findings we determined mRNA expression of IFN-gamma and IL-1 beta, considering their participation in the pro-inflammatory process that induce neurodegeneration. For IFN-gamma factorial ANOVA showed an interaction between diet and sex ($F_{(1,56)}$= 5.01, p=0.0292). Analysis of simple effects indicated that there is an effect of diet in males ($F_{(1,56)}$= 12.29, p<0.0009) but not in females ($F_{(1,56)}$= 0.06, NS). Post-hoc test showed that in males fed with HFD and exposed to stress, a significant increase of IFN-gamma was observed but not in females (Fig 5D). For IL-1 beta factorial

ANOVA indicated an interaction diet x sex ($F_{(1,56)}$= 4.54, p<0.0375) and diet x stress ($F_{(1,56)}$= 6.55, p<0.0132). Analysis of simple effects showed that there is an effect of CMS in males fed with CD ($F_{(1,56)}$= 17.10, p<0.0001) but not with HFD $F_{(1,56)}$= 0.05, NS). Post-hoc analysis indicated a decrease of IL-1 beta in animals fed with HFD, and fed with CD exposed to stress (Fig 5 E).

3.5. Impaired cognitive performance in males correlated with a reduction in brain-derived neurotrophic factor mRNA expression in spleen.

In order to propose if peripheral lymphocytes could be used as biomarkers to detect central nervous system events, we determined mRNA expression of BDNF, NT-3, NGF, IFN-gamma and IL-1 beta in the spleen, as representative immune organ in mice. As can be seen in Fig. 6 A, for BDNF mRNA levels in spleen were similar to those found in the hippocampus. Kruskal-Wallis analysis showed significant differences between groups for BDNF mRNA levels in spleen (KW H (7) =42.60, p<0.0001). As can be seen in Fig 6 A males fed with HFD had a decrease of BDNF that is significant in animals exposed to CMS. No differences were observed in male animals fed with CD and exposed to CMS compared to the non-exposed controls and in females. In addition, for NGF mRNA levels in spleen factorial ANOVA indicated an effect of stress exposure ($F_{(1;56)}$=6.57, *p*=0.013). However, post-hoc analysis did not shown changes between experimental groups (Fig. 6 B). Non-detectable levels of NT3 were found in the spleen. Concerning to mRNA expression of INF-gamma in spleen, ANOVA showed an interaction sex x diet ($F_{(1,56)}$= 6.58, p=0.0130). Simple effect analysis indicated an effect of diet in males ($F_{(1,56)}$=8.00, p=0.0065). Post-hoc analysis did not show significant differences between groups (Fig 6 C). For IL-1 beta factorial ANOVA did not show any significant effect (Fig 6 D).

To analyze the ability of peripheral BDNF to predict the levels of BDNF in the hippocampus, we performed a Spearman correlation analysis. As can be seen in Fig 7 A significant positive correlation was observed between spleen and hippocampal BDNF levels (r= 0.3647, *p*=0.0031).

In addition, we analyzed the relationship between behavior and BDNF levels, using correlation analysis to assess the ability of central and peripheral BDNF to affect spontaneous alternation. Pearson correlation analysis shows a significant positive correlation between hippocampal BDNF mRNA and spontaneous alternation (r= 0.8148, *p*<0.0001) (Fig 7 B). Moreover, using Spearman correlation analysis a positive correlation was found between spleen BDNF mRNA and spontaneous alternation (r= 0.2761, *p*=0.0272) (Fig 7 C).

4. Discussion

This study describes the long-term effects of HFD in combination with CMS stress exposure on metabolic and cognitive parameters in C57Bl/6J male and female mice. Results indicated that males present alterations in spatial memory associated to changes in hippocampal and spleen BDNF levels.

A daily consumption of HFD, in which 60 kcal% was attributed to fat, was replicated in C57Bl/6J mice analogously to the onset of diet-induced obesity in humans as a chronic disorder characterized by a time-dependent progressive disease (Collins et al., 2004). To resemble human habits, mice were fed with HFD from one-week post-weaning to three months of age encompassing adolescence (from weaning to adulthood). At his period, several crucial biological events occur such as body growth, hormonal changes, where brain and immune system are in the developmental transition. (Spear, 2000; Hammelrath et al., 2016; Holladay and Smialowicz 2000). On the other hand, it has been described that

chronic stress-induced alterations in eating behaviors can lead to the development of obesity (Bartolomucci et al., 2009; Patterson and Abizaid, 2013; Balsevich et al., 2019, Wei et al., 2019) or, on the other hand, to anorexia (Michel et al., 2005; González-Torres and Dos Santos, 2019; Finger et al., 2012). To study the effect of chronic stress in the alterations induced by HFD, adult mice (three months of age) were exposed to CMS mild stress for 20 weeks. This period was chosen because, in humans, adulthood is a time when exposure to modern life stressors is more frequent.

In this model, we observed that after 28 weeks with a HFD regimen male and female mice exhibited increased body weight respect to age-matched mice fed the control diet. This weight gain was accompanied by an increase in abdominal and subcutaneous fat pads that correlates with an increment in caloric intake. Stress exposure induced a decrease in body weight, related to a decrease in caloric efficiency in both males and females. Interestingly, under stress, a decrease of VF and SF pad was observed in females fed with HFD and only SF but not VF decrease occurred in males fed with HFD. Danforth (2000) proposed the 'adipose tissue expandability hypothesis' which posits that there is a limited capacity of subcutaneous adipocyte lipid storage. Once this limit is reached, the lipid excess is deposited in undesirable or ectopic fat depots, such as visceral adipose tissue, liver and skeletal muscle, which are associated with insulin resistance and metabolic dysfunction. In this context, female mice exposed to CMS would have a lower risk of suffering metabolic disease under HFD nutrition.

Following these results, we observed a significantly higher level of glycemia after 2 hours of glucose administration in male mice following stress exposure, HFD-feeding and both. Interestingly, males fed with HFD and exposed to CMS have a significant fold change of glucose levels respect to CD and HFD alone. Nevertheless, although HFD feeding in females increased body weight, this did not lead to alterations in 2hPG glucose

levels. In addition, a significant increase in basal insulin levels was observed in female under HFD. In general, body weight, blood glucose and insulin levels are higher in males than in females. Therefore, these results showed that HFD feeding influence glucose metabolism depending on sex, showing in males a worse glucose metabolism profile than in females. It was reported that the characteristics of obesity induced by HFD feeding were influenced by the sex, strain, and age of the mice (Nishikawa et al., 2007). In particular, previous studies have discovered sex differences in C57Bl/6 mice under nutritional overload (Pettersson et al., 2012; Arcones et al., 2019). In general, it was described that young female mice are more resistant than young males to develop metabolic alteration (adipose tissue inflammation, glucose intolerance, and hyperinsulinemia) induced by HFD. This female-specific protection was lost in more aged females, which is mostly attributed to altered estrogen levels after menopause (Arcones et al., 2019). Finger et al. (2012) found that male C57BL/6J mice subjected to chronic psychosocial stress and a HFD for six-week treatment have a pronounced inhibition of body weight gain, accompanied by reduced caloric intake and caloric efficiency. But, in mice on a low-fat diet, exposure to stress for three weeks caused an increase in weight gain, caloric intake, and caloric efficiency. However, Castañeda et al. (2011) described that previous chronic variable stress exposure induces a decrease in body weight in male C57BL/6J mice regarding feeding with a standard diet, but it does not affect obesity onset under a HFD feeding. However, stress exposure induced impaired glucose tolerance in animals fed with a HFD. These results highlight the importance of considering the chronic aspects of diet and stress and their time-dependent interplay (Finger et al., 2012).

Concerning lipid metabolism, HFD causes an increase in total cholesterol, which is associated with HDL particles in males fed HFD and females fed with HFD and exposed to CMS. Moreover, no change in circulating levels of TG was observed in mice fed with HFD.

Biddinger et al., (2005) have previously reported that C57BL/6 mice fed with HFD decrease their triglyceride values despite their insulin-resistant state. It was proposed that the increased fat intake dwindled the circulating triglycerides either by suppressing TG production and/or increasing TG clearance (Guo et al., 2009). Similar results were obtained by Ferell et al. (2016). Nonetheless, other authors showed that HFD induced an increase in TG, total cholesterol, and a decrease of HDL-cholesterol (Tan et al., 2014). It seems that the HFD always leads to dysregulation of glucose metabolism with insulin resistance, but lipid dysregulation is more variable. Our results indicate that in our C57Bl/6J-HFD model, the impairment of glucose metabolism prevails.

In respect to the effects of a HFD and/or CMS exposure on cognitive performance, we also observed differences between males and females. In the spontaneous alternation task, the percentage of alternation decreased in males fed with HFD, exposed to stress, or who experienced both. None of these effects were present on female mice. Similarly, in the Barnes test, only male mice exposed to HFD and CMS were affected in the latency to finding the target hole. Learning and memory of spatial and contextual information depend on the hippocampus. Our results indicate that stress and/or diet induced a hippocampal-dependent spatial memory deficit, however, this deficit was not observed in the open field task, which evaluates non-associative contextual memory. This memory is one of the most elementary forms of learning; further impairment of the normal physiology of the hippocampus may be necessary to observe any alteration (Miller and Grace, 2012).

It has previously been described that insulin and HFD-induced insulin resistance is involved in the spatial memory in mice (McNay et al., 2010; Vinuesa et al., 2016). Our results within our model do not seem to suggest that there could be a direct relationship between insulin resistance and cognitive performance. However, more studies are necessary to evaluate the role of insulin resistance in this model.

Taking into account that neurotrophins play an essential role in the regulation of neurocognitive functions, we studied neurotrophin mRNA expression in the hippocampus. Results indicated that males fed with HFD had a reduction in BDNF mRNA expression. It was described that BDNF is implicated in learning and memory formation and it has an important role in synaptic plasticity that contributes to cognitive processes (Bekinschtein et al., 2007). In accordance with our results, Park et al. (2010) described that HFD impairs hippocampal neurogenesis related to a decrease in BDNF in male C57BL/6 mice.

In addition, it was shown that low grade chronic systemic inflammation is generated during obesity, that in turn cause changes in the brain and induce neuroinflammation (Salas-Venegas et al., 2022). Of particular interest in the context of obesity-induced cognitive decline is the neuroinflammation generated in the hippocampus. For this reason, we determined IFN-gamma and IL-1 beta in hippocampus considering their participation in the pro-inflammatory process that induce neurodegeneration. In males, our results indicated a significant increase of INF-gamma due HFD feeding and stress exposure. For Il-1 beta results displayed a decrease related to HFD and/or stress exposure. Non changes were observed at peripheral levels. Many reports support the role of IL-1 beta in spatial recognition and contextual learning (Bourgognon and Cavanagh, 2020). It was reported that IL-1 beta can also modulate plasticity and memory by promoting the production of neurotrophic factors (Bourgognon and Cavanagh, 2020). However, at pathophysiological levels, IL-1 beta can produce detrimental effects on memory (Bourgognon and Cavanagh, 2020). Goshen et al. (2007) demonstrated that IL-1 beta in hippocampal-dependent memory follows an inverted U-shaped pattern, a slight increase of IL-1 beta in brain improves memory, while both, the excess or the decrease, results in impaired memory. Concerning to IFN-gamma, it has been shown that high levels of this cytokine in the brain lead to reactive gliosis, hypomyelination, and macrophage/microglial

stimulation contributing to neuronal cell death (Colton, 2009). Taking into account these findings, it is possible that the increase of IFN-gamma induced by HFD and/or the decrease of IL-1 beta lead to cognitive impairment, in particular in the Barnes Maze test that involve a training for learning and spatial memory. However, no changes of these cytokines were found at systemic level.

It was proposed that peripheral leukocytes could be used as biomarkers to detect central nervous system events. In particular, it was found that BDNF mRNA expression could be a biological marker indicating the severity of executive dysfunctions in patients with substance-use disorders (Anders et al., 2020). Similarly, spleen has been used as representative immune organ in mice in the same way as the use of peripheral blood mononuclear cells in humans (Sun et al., 2019). In this sense, we found that HFD feeding induces cognitive alterations related to a decrease in BDNF mRNA expression in spleen.

An evident question that these results raise is how the peripheral levels of BDNF could predict brain levels. According to Pan and colleagues (1998) most members of the mammalian NT family appear to cross the blood-brain barrier through saturable transport systems to essentially arrive intact in the central nervous system (CNS). Although it was shown that the exogenous administration of these neurotrophins as a therapeutic in neurodegenerative diseases is limited by a lack of blood-brain-barrier permeability, poor half-life, and rapid degradation (Houlton et al., 2019). There is evidence showing that aerobic exercise appears to increase circulating levels of neurotrophins, particularly BDNF, and promote neurocognitive improvement (Lippi et al., 2020; Ribeiro et al., 2021). It was reported that the brain contributed for up to 70–80% of the circulating BDNF, being the major but not the sole contributor to circulating BDNF (Rasmussen et al., 2009). It is of particular interest to understand the cross-talk of neurotrophins between the brain and periphery. In this sense, the possible existence of a muscle-brain crosstalk associated to

physical activity was suggested (Pedersen, 2019). Although it is not fully elucidated how the peripheral levels of BDNF could be predictive of brain levels, there is significant evidence that relates peripheral BDNF with cognitive performance. In accordance, our results indicate a significant positive correlation of BDNF mRNA expression levels between central and peripheral tissues. Moreover, a correlation between spatial working memory and both the hippocampal and spleen levels was observed.

5. *Conclusion*

Our results indicate that males are more sensitive to the deleterious effects of diet and stress, producing more detrimental effects in both metabolic and spatial working memory. Cognitive deficits are related to both a central and peripheral decrease in BDNF mRNA expression. Further studies are necessary to evaluate if peripheral BDNF mRNA expression could predict the level of cognitive performance in individuals with diet-induced obesity.

6. Strengths and Limitations of the Study

To our knowledge, this is the first study that suggests peripheral BDNF mRNA expression as predictor of cognition in individual with diet-induced obesity. The model used in this study resembles the situation in humans. Mice were fed with HFD one-week post-weaning, at an age that coincides with the beginning of adolescence, where eating and sleeping are in the developmental transition. To study the effect of chronic stress in the alterations induced by HFD, the period chosen (three to eight months of age) was because adulthood is a time when exposure to modern life stressors is more frequent. Another strength of the study is the inclusion of sex as a biological variable as the National Institutes of Health (NIH) encouraged for research designs (NIH, 2021).

On the other hand, this work has some limitations. Concerning the HFD that was used, it was prepared by mixing butter and lard into the control diet after its formulation, so the composition of macro and micronutrients was not the same in the CD and HFD diets. However, as explained in materials and methods, both were in accordance with the composition and concentration recommended for adult maintenance in the AIN-93 purified diets for laboratory rodents (Reeves et al., 1993).

Finally, the measurement of neurotrophins and cytokines mRNA expression in spleen is not a direct analysis of this expression in peripheral blood mononuclear cells. Although spleen measurements may be a first step to the biomarker question, more studies determining neurotrophins on peripheral blood mononuclear cells will be necessary to be able to properly propose them as a biomarker of cognitive impairment in individuals with diet-induced obesity.

Credit authorship contribution statement

A M Genaro and M R Wald: designed and planned the research and wrote the manuscript, A Prochnik carried out the animal study and performed the experiments, M R Gonzalez Murano and M P Marcone collaborate in the experiments A L Burgueño performed statistical analysis, graphics and collaborate in mRNA expression analysis. MR Rubinstein collaborated in statistical analysis and English improvement. M S. Bianchi performed determination of insulin levels. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest

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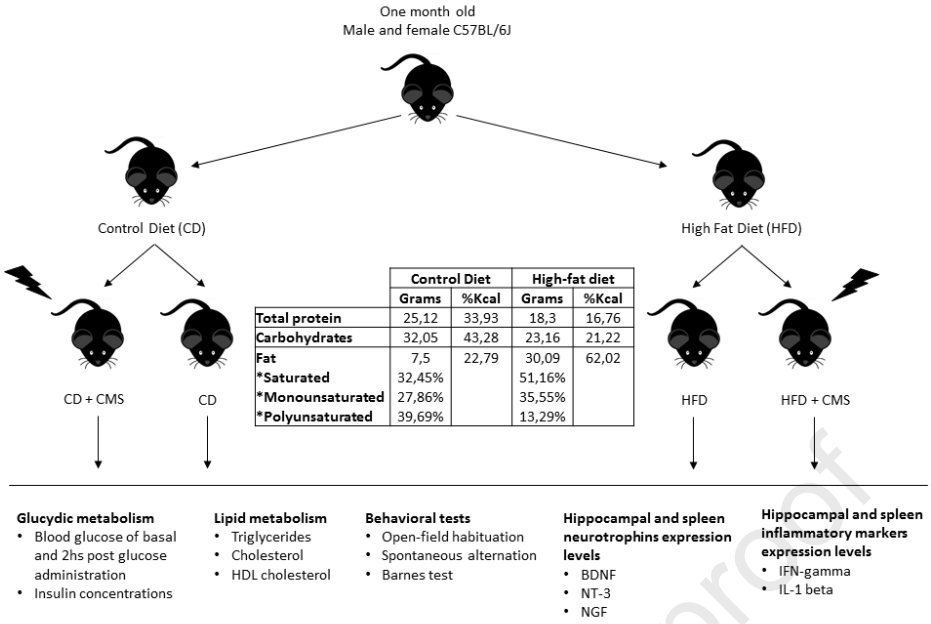
Table 1
Primers used for the mRNA gene expression by RT-PCR.

Gene	Sequence	Amplicon size	Tº Annealing
BDNF FWD	5’ CTG AGC GTG TGT GAC AGT ATT A 3’		
BDNF REV	5’ CTT TGG ATA CCG GGA CTT TCT C 3’	112 pb	60ºC
NGF FWD	5’CAG TGA GGT GCA TAG CGT AAT 3’		
NGF REV	5’ CTC CTT CTG GGA CAT TGC TAT C 3’	107 pb	60ºC
NT3 FWD	5’ CCT GGA AAT AGT CAC ACG GAT G 3’		
NT3 REV	5’ CTT GGA TGC CAC GGA GAT AAG 3’	115 pb	60ºC
IFN-gamma FWD	5’ TGC TGA TGG GAG GAG ATG TCT AC 3’		
IFN-gamma REV	5’ ACC TGA TAC ATT CGA GTG CTG T 3’	76 pb	60º
IL-1 beta FWD	5’ GAG GAC ATG AGC ACC TTC TTT 3’		
IL-1 beta REV	5’ GCC TGT AGT GCA GTT GTC TAA 3’	121 pb	60º
GAPDH FWD	5’ CGT CCC GTA GAC AAA ATG GT 3’		
GAPDH REV	5' GAA TTT GCC GTG AGT GGA GT 3’	177 pb	60ºC



	Control Diet		High-fat diet	
	Grams	%Kcal	Grams	%Kcal
Total protein	25,12	33,93	18,3	16,76
Carbohydrates	32,05	43,28	23,16	21,22
Fat	7,5	22,79	30,09	62,02
***Saturated**	32,45%		51,16%	
***Monounsaturated**	27,86%		35,55%	
***Polyunsaturated**	39,69%		13,29%	

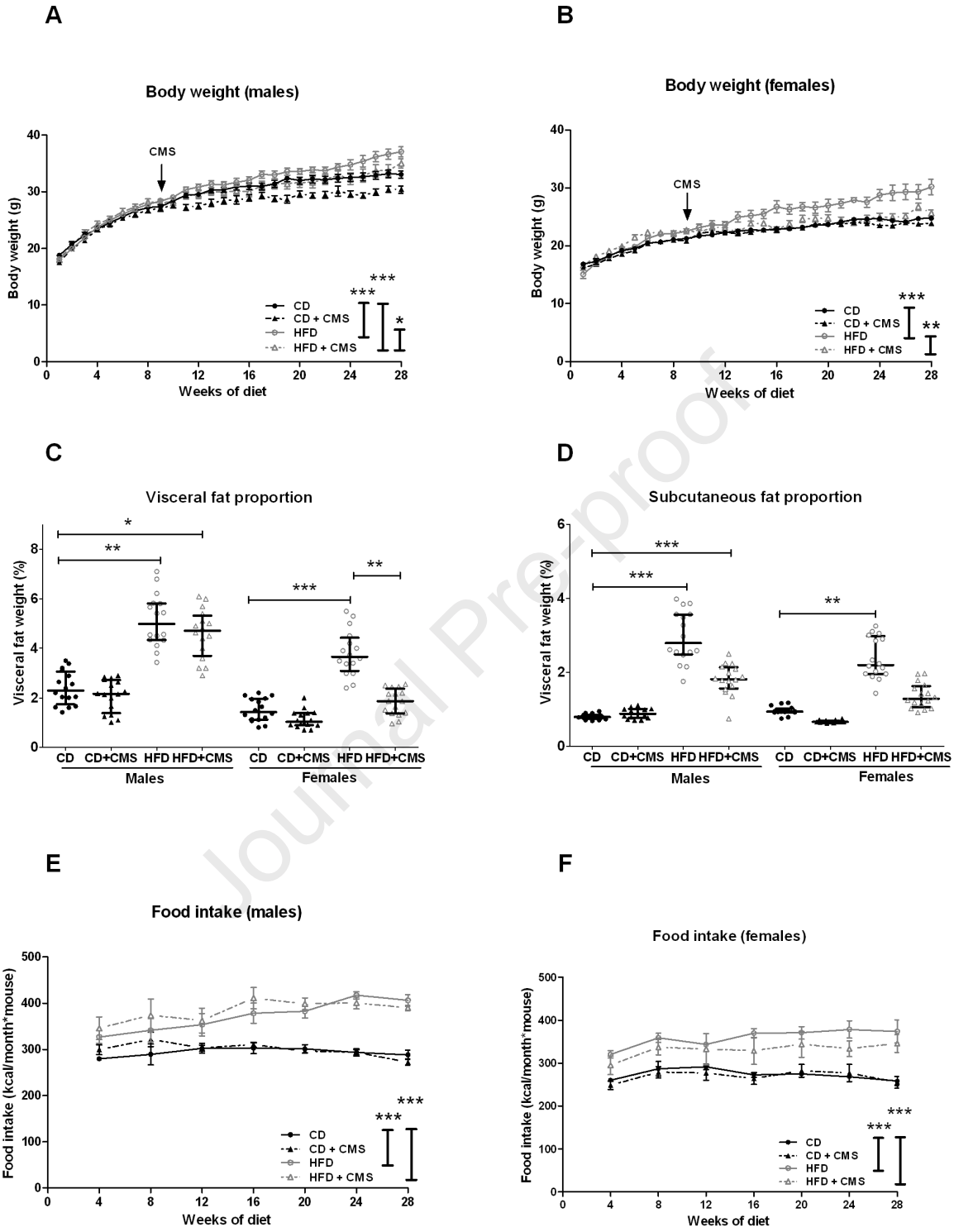
Glucydic metabolism
- Blood glucose of basal and 2hs post glucose administration
- Insulin concentrations

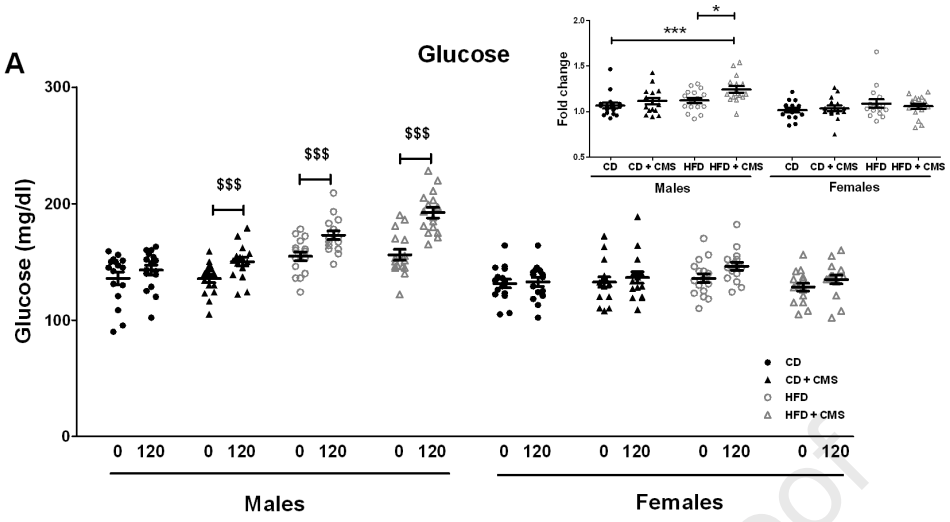
Lipid metabolism
- Triglycerides
- Cholesterol
- HDL cholesterol

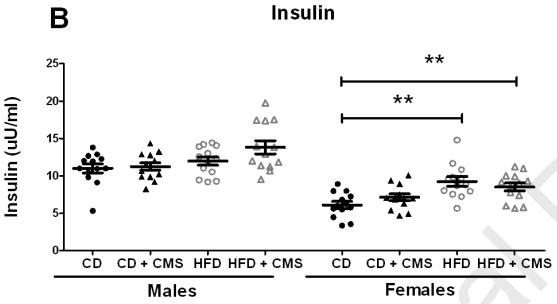
Behavioral tests
- Open-field habituation
- Spontaneous alternation
- Barnes test

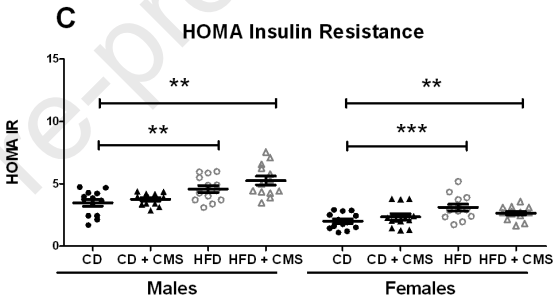
Hippocampal and spleen neurotrophins expression levels
- BDNF
- NT-3
- NGF

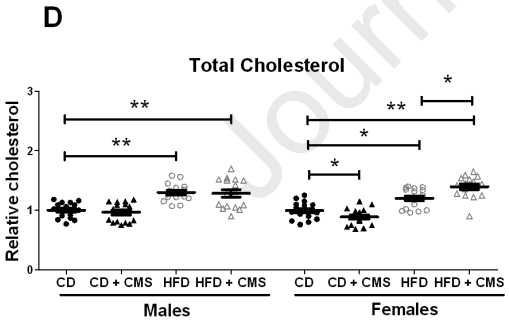
Hippocampal and spleen inflammatory markers expression levels
- IFN-gamma
- IL-1 beta

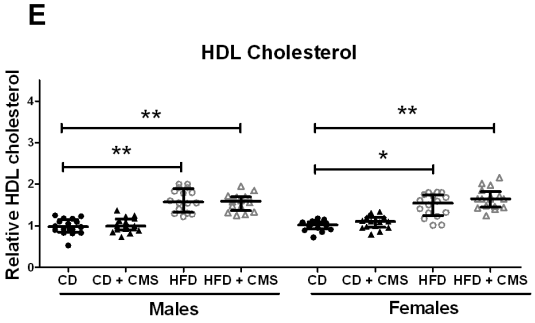


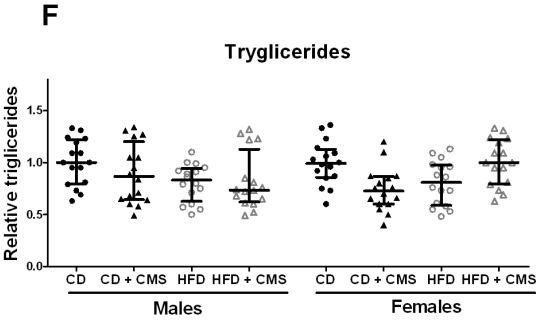


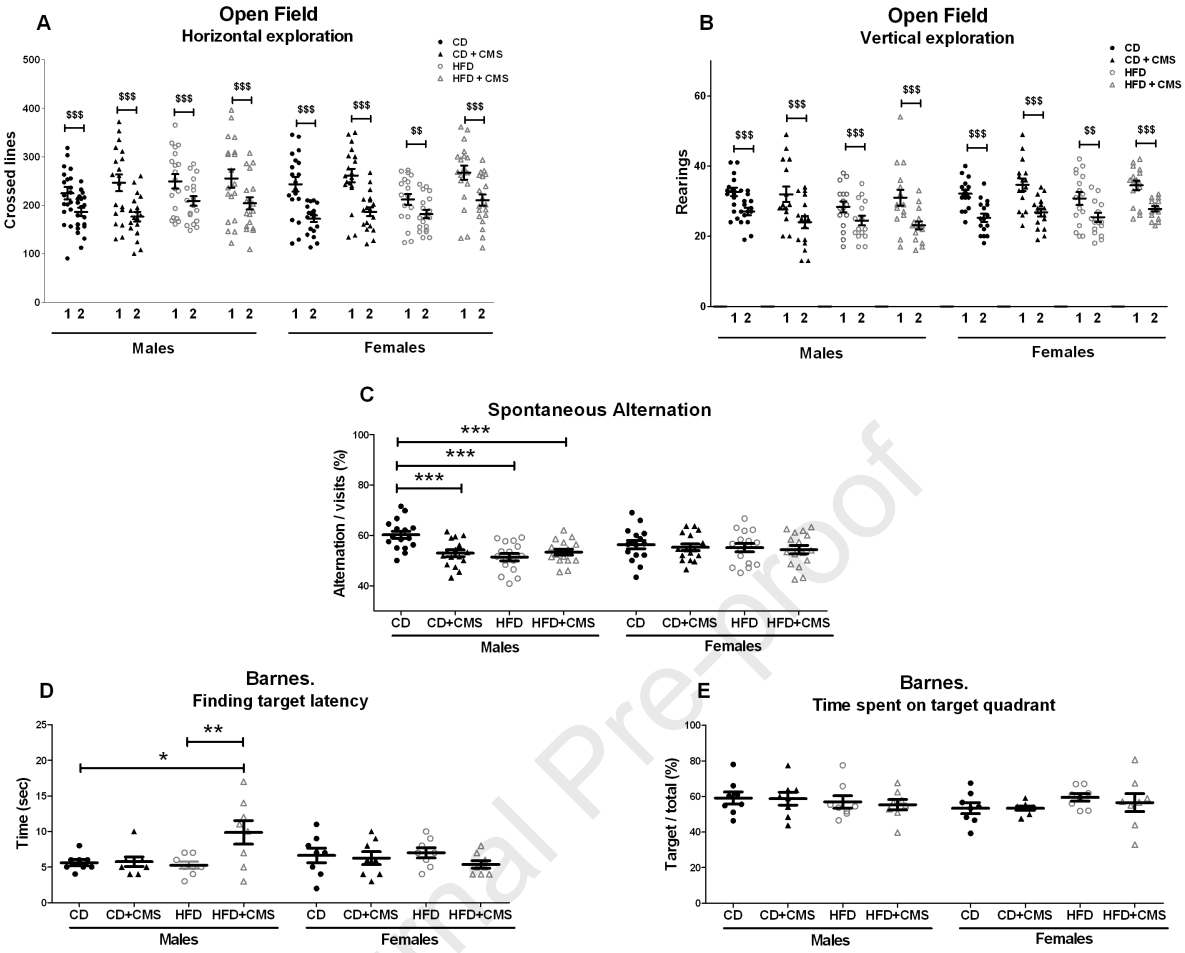


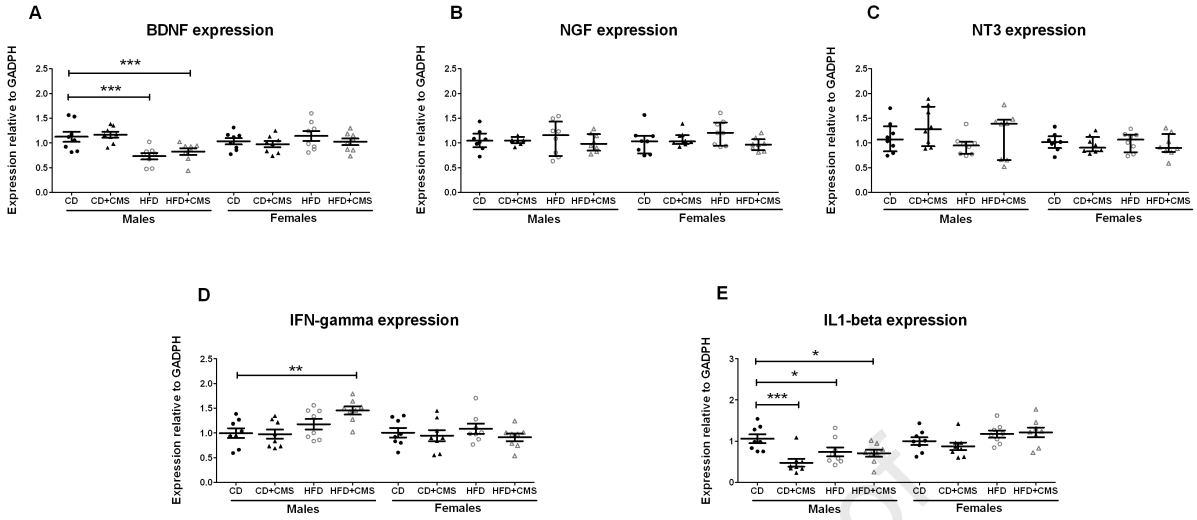


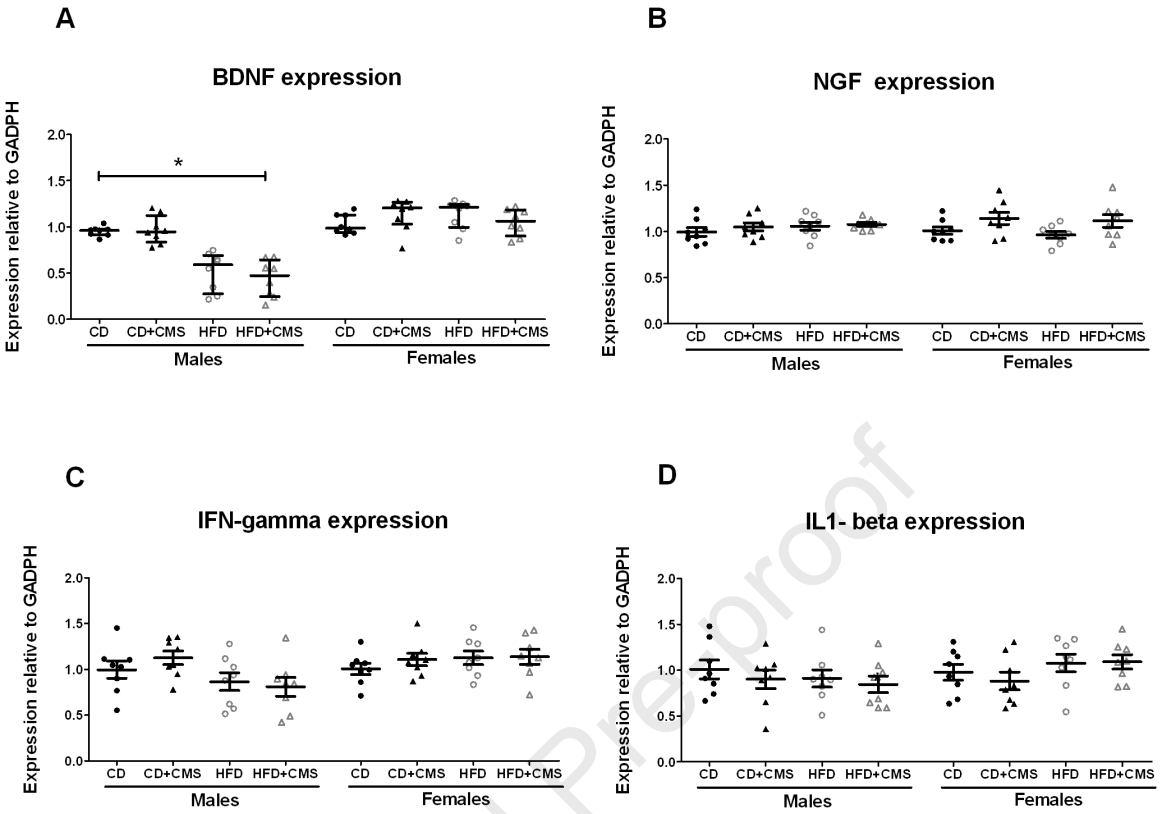












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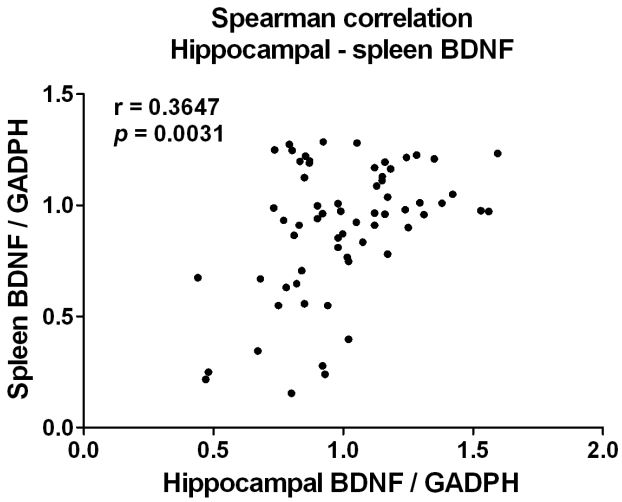

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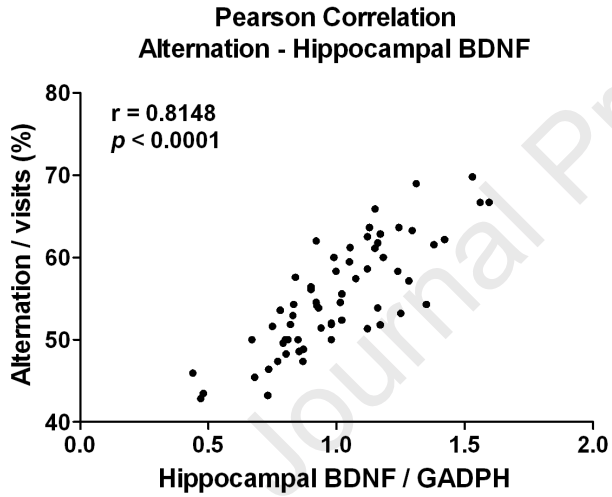

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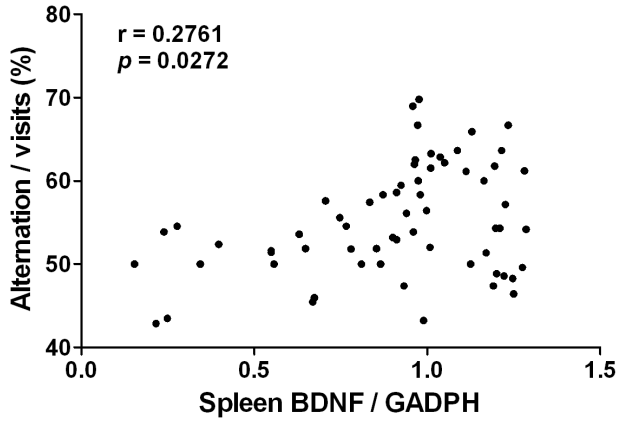

Highlights

High-fat diet and/or stress led to impaired glucose metabolism and cognitive function.

Cognitive deficit under high-fat diet and/or stress were worse in males than females.

Cognitive deficit was linked to central and peripheral fall in BDNF mRNA expression.

Lymphocyte BDNF mRNA expression as possible marker of cognitive deficit in overweight.

Credit authorship contribution statement

A M Genaro and M R Wald: designed and planned the research and wrote the manuscript, A Prochnik carried out the animal study and performed the experiments, M R Gonzalez Murano and M P Marcone collaborate in the experiments A L Burgueño performed statistical analysis, graphics and collaborate in mRNA expression analysis. MR Rubinstein collaborated in statistical analysis and English improvement. M S. Bianchi performed determination of insulin levels. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest