INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

High-fat diet impairs short-term memory in mice

Tesis que presenta

Eder Oswaldo Portillo Gerónimo

Para obtener el grado de

Maestro en Ciencias en Biología Molecular

Codirectores de la Tesis:
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Constancia de aprobación de la tesis

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Créditos Institucionales

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MAESTRO EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Eder Oswaldo Portillo Gerónimo

sobre la Tesis intitulada:

High-fat diet impairs short-term memory in mice

que se desarrolló bajo la codirección de

Dr. Cesaré Moisés Ovando Vázquez
Dr. J. Sergio Casas Flores

El Jurado, después de deliberar, determinó

APROBARLO

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Dedicatorias

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Contenido

Resumen

La dieta alta en grasa afecta la memoria a corto plazo en ratones

Una dieta con un aporte excesivo de calorías, grasas, azúcares y baja en fibra dietética, genera trastornos metabólicos que conducen a enfermedades como la obesidad. Dicha dieta también puede generar un desequilibrio de la microbiota intestinal, conocido como disbiosis. La disbiosis inducida por una dieta alta en grasa (DAG) se ha relacionado con enfermedades neurológicas, alteraciones del estado de ánimo y del comportamiento, a través del eje intestino-cerebro. En este trabajo se indujo un modelo de obesidad mediante DAG (al 60% de las kcal) durante 16 semanas en ratones machos y hembras de 8 semanas de edad de la cepa C57BL6/J. Los marcadores metabólicos: glucosa, triglicéridos y colesterol no se vieron afectados negativamente. Para evaluar el efecto de DAG en la memoria a corto plazo, se realizó la prueba de reconocimiento de objetos novedosos (PRON) al final del experimento. Al concluir la DAG, se observó un aumento significativo en la ganancia de masa corporal en machos, que no se detectó en las hembras, en comparación con sus controles alimentados con una dieta estándar. Además, se determinó una disminución significativa en el tiempo de exploración NORT en los machos, pero no en las hembras. Nuestros resultados indican que existe una correlación entre la obesidad y alteración de la memoria a corto plazo en los machos.

PALABRAS CLAVE. Obesidad, Disbiosis, PRON

Abstract

High-fat diet impairs short-term memory in mice

A diet containing an excessive calorie intake, fat, sugar, and low in dietary fiber, generates metabolic disorders leading to obesity, which also can generate an imbalance of the gut microbiota, known as dysbiosis. Dysbiosis induced by a high-fat diet (HFD) has been related to the development of neurological diseases, mood swings, and behavioral alterations, through the gut-brain axis. In this work, an obesity model was induced by the intake of a HFD (at 60% of kcals) during 16 weeks in 8-week-old male and female mice of the C57BL6/J strain. According to the results, metabolic markers: glucose, triglycerides and cholesterol were not negatively affected. To assess the effect of HFD in short-term memory, the novel object recognition test (NORT) was performed at the end of the experiment. After treatment with DAG, we observed a significant increase in body mass gain with respect to control groups, this increase was more marked in male mice than in females. Furthermore, a significant decrease in NORT exploration time was determined in males, but not in females. Our results indicate that there is a correlation in between obesity and impairment in short-term memory in males.

KEYWORDS. Obesity, Dysbiosis, NORT

High-fat diet impairs short-term memory in mice

Introduction

Obesity and overweight are an abnormal or excessive accumulation of body fat. Obesity is caused mainly by environmental factors such as bad nutrition. It has been shown that high-calorie diets, including high-fat diet and high-sugars diets, increase the probability of cognitive deterioration[1–4]. In human adults (20-40 years old), overweight is diagnosed using the Body Mass Index (BMI) ranging from 25 to 29.9 Kg/m^2, whereas obesity is diagnosed with a BMI of 30 Kg/m or higher[2 5]. Obesity is considered a major public health problem and its prevalence has increased in the last years worldwide[6]. In 2020, Mexico reported an incidence of obesity of 31.5% and 40.2% for men and women over 20 years old, respectively[6,7]. Epidemiological studies have shown that a high BMI is a risk factor for chronic diseases, such as type 2 diabetes, cardiovascular disease, chronic kidney disease, systemic arterial hypertension, various types of site-specific cancers, and musculoskeletal disorders[8].

The gut microbiota is a complex ecosystem of microorganisms comprised of bacteria, viruses, protozoa, and fungi that modulates its host physiology. In the gut microbiota, bacteria are the predominant taxonomic group, which has been estimated to be equivalent of the number to cells in the human body[9]. The composition of the gut microbiota is dynamic, and depends on its host factors such as age, genetics, environmental and external factors (i.e., use of drugs), physical activity and diet[10]. There is growing evidence suggesting that a high-fat diet (HFD), comprised of more than 30% of the total energy of fat, can provoke quantitative and qualitative imbalances in the constitution of the gut microbiota, also known as dysbiosis. Dysbiosis is closely related to the development of metabolic disorders, including obesity, type 2 diabetes, high blood pressure, systemic inflammation, dyslipidemia, and other diseases[11]. There is strong evidence that HFD promotes changes in the composition of the microbiota in animal models. For instance, HFD led to an increase in gram-negative bacteria and a decrease in gram-positive bacteria[12–16].

Interestingly, studies in animals and humans have shown that gut microbiota is associated with the central nervous system function[17–20]. In recent years, emerging evidence proposes a bidirectional connection between the gut microbiota and the brain, the so-called “gut microbiota-brain axis,” which plays an important role in the modulation of cognitive functions and behavior[21–25]. Numerous studies have demonstrated that the microbiota plays a pivotal role in neurological disorders such as multiple sclerosis, autism, Parkinson’s, and Alzheimer’s disease[26–32]. In animal models, HFD can affect spatial memory and behavior in mice and rats, respectively[33,34]. In this regard, obesity, HFD and dysbiosis can led to a decrease in the concentration of short-chain fatty acids (SCFA) produced by the intestinal microbiota. SCFA and butyrate are considered beneficial for brain and cognitive activities such as learning, memory, and associative memory[35–37].

Here we focus on understanding whether obesity may contribute to short-term memory deterioration in an obesity C57BL6 mouse model, including 12-week-old males and females. In this study, we fed mice with 60% HFD or control diet for 16 weeks. Body weight, glucose, triglycerides, and total cholesterol were measured every 4 weeks. Subsequently, the evaluation of behavior was carried out through the novel object recognition test (NORT).

Results

HFD leads to increase mice body weight. To induce obesity, mice were fed with a high-fat diet (HDF) for 16 weeks while control group were fed with a standard control diet (SCD), as shown

in Figure 1. After 16 weeks of HFD, mice showed significantly, α=0.1, higher body weight (two-way ANOVA test showed effect on time per treatment, p = 0.0059) (FIG. 1A). Body weight gain was affected according to sex, since we saw that males gained more body weight than females (Figure 2B and Table 1). Body weight increase may be explained because HFD-fed male and female mice consumed higher energy intake than the control groups (Fig. 2C).[38] These results are consistent with what is expected in models of obesity in mice[38].

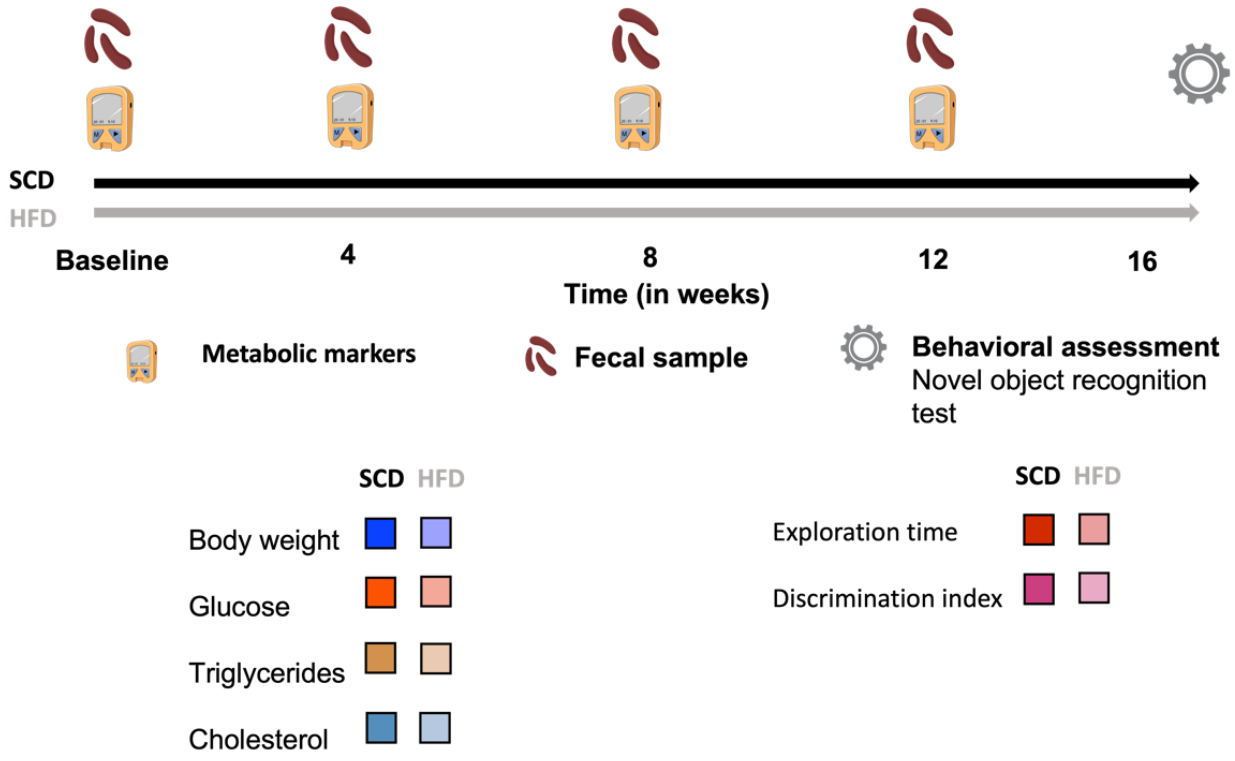

Figure 1. Schematic of the experimental design showing the trial groups, mice fed High Fat Diet (HFD) and the Standard Control Diet (SCD). The timeline shows the weeks in which metabolic markers (glucose, triglycerides, cholesterol, and body weight) and stool samples were collected at the end of the novel object recognition test was performed. Below, the treatment key color is depicted

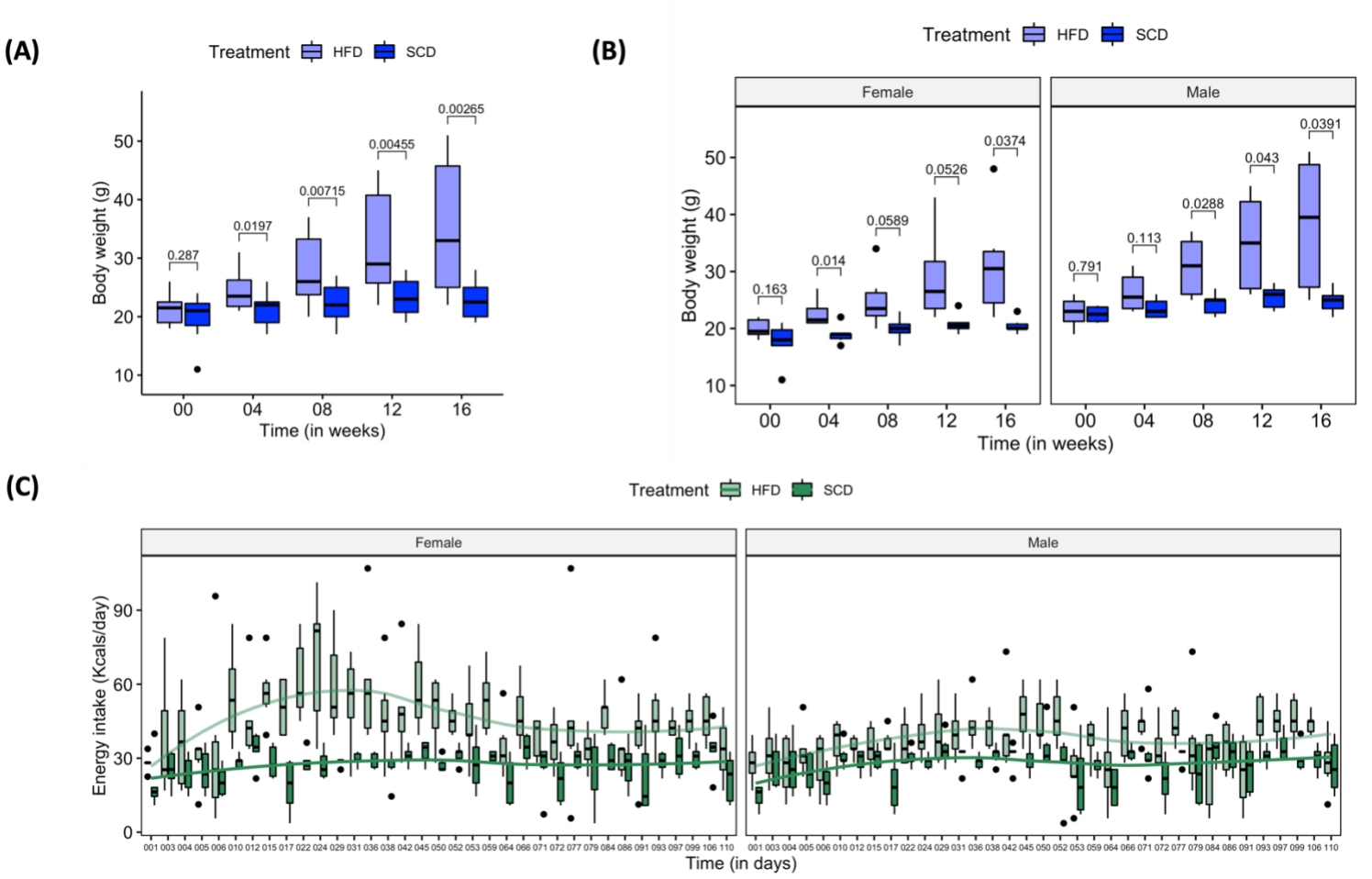

Figure 2. Body weight tracking in male and female mice. A) Body weight in grams between experimental groups over time. B) Body weight in grams between experimental groups by sex over time. C) Energy intake (Kcals) by sex. SCD (in dark green) and HFD (in light green). The numbers above represent p-values obtained from t-test analysis between experimental groups.

HFD did not affect metabolic markers but increased visceral fat accumulation. Metabolic markers including blood glucose, total cholesterol, and triglycerides were measured every 4 weeks to determine metabolic changes. However, substantial alterations were not observed in the different evaluations (two-way ANOVA test, *p*-values=0.131, *p*-values=0.140, *p*-values=0.971, respectively). All-metabolic markers remained within the health interval in mice (Fig 3 A-C, and table 1). On the other hand, visceral fat increased substantially in males and females fed with HFD (Fig 3D), which correlates with an increased body weight. These results support that obesity was induced.

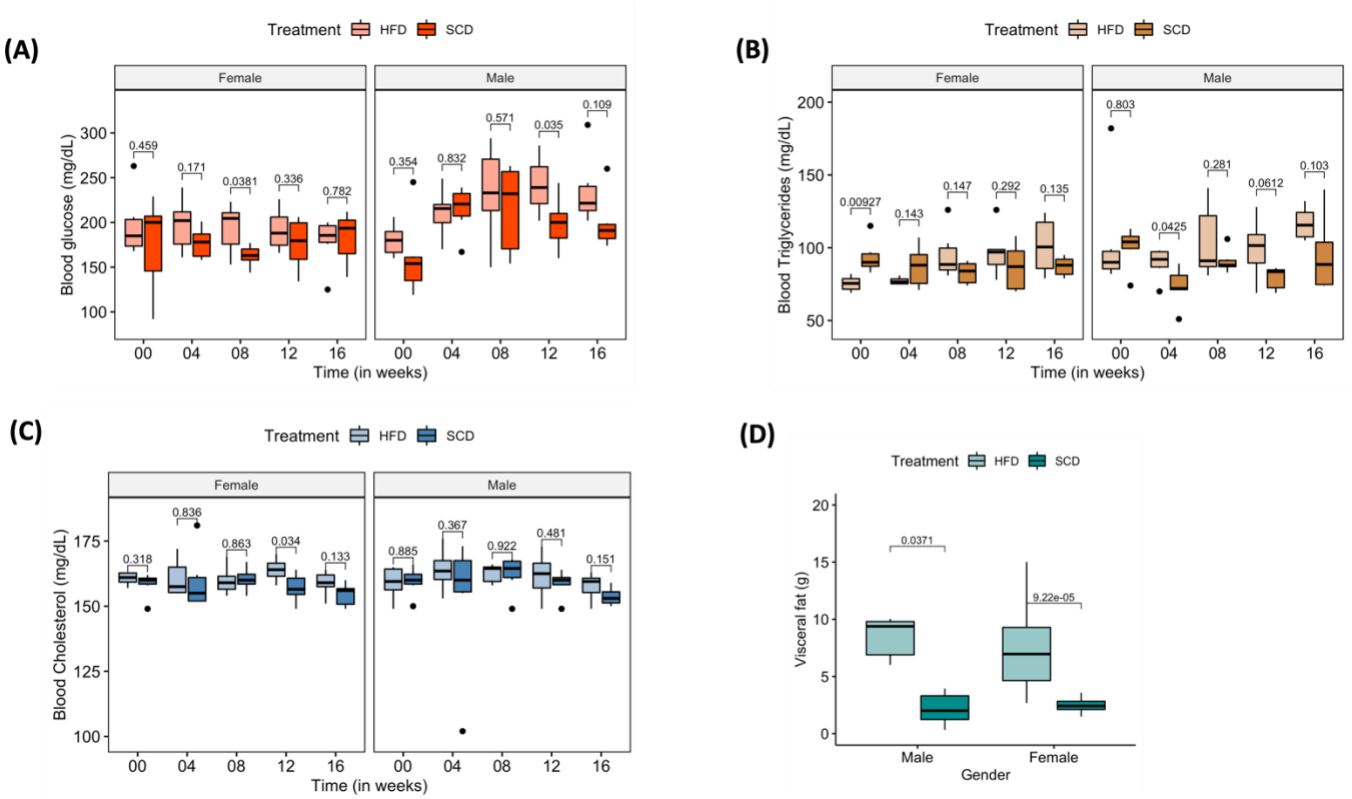

Figure 3. Metabolic markers and visceral fat in male and female mice. Mice were fed for 16 weeks with HFD or with SCD. A) Glucose concentration in mg/dL. B) Triglycerides in mg/dL. C) Cholesterol in mg/dL. D) Visceral fat in grams. SCD (in dark color) and HFD (in light color). Numbers above represent *p*-values obtained from t-test analysis between experimental groups.

HFD impaired mice's short-term memory. Mice performance in the NORT was used to evaluate the effect of HFD on short-term memory (Figure 1). Exploration time with the novel object (novel) decreases significantly in HFD group (*p*-value=0.006), (Fig. 4A). Males were significantly, α=0.1, affected (*p*-value=0.020) but not females (*p*-value=0.242) during the exploration time (Fig. 4B). HFD treatment negatively affected novel object exploration preference (*p*-value=0.886) in males, not the case for females (*p*-value=0.045), (Fig. 4C). Furthermore, changes in discrimination index (DI) were appreciated in males (*p*-value=0.080), but not in females (*p*-value=0.884), (Fig. 4D). These findings suggest that short-term memory could be impaired in HFD-fed males, but not in females.

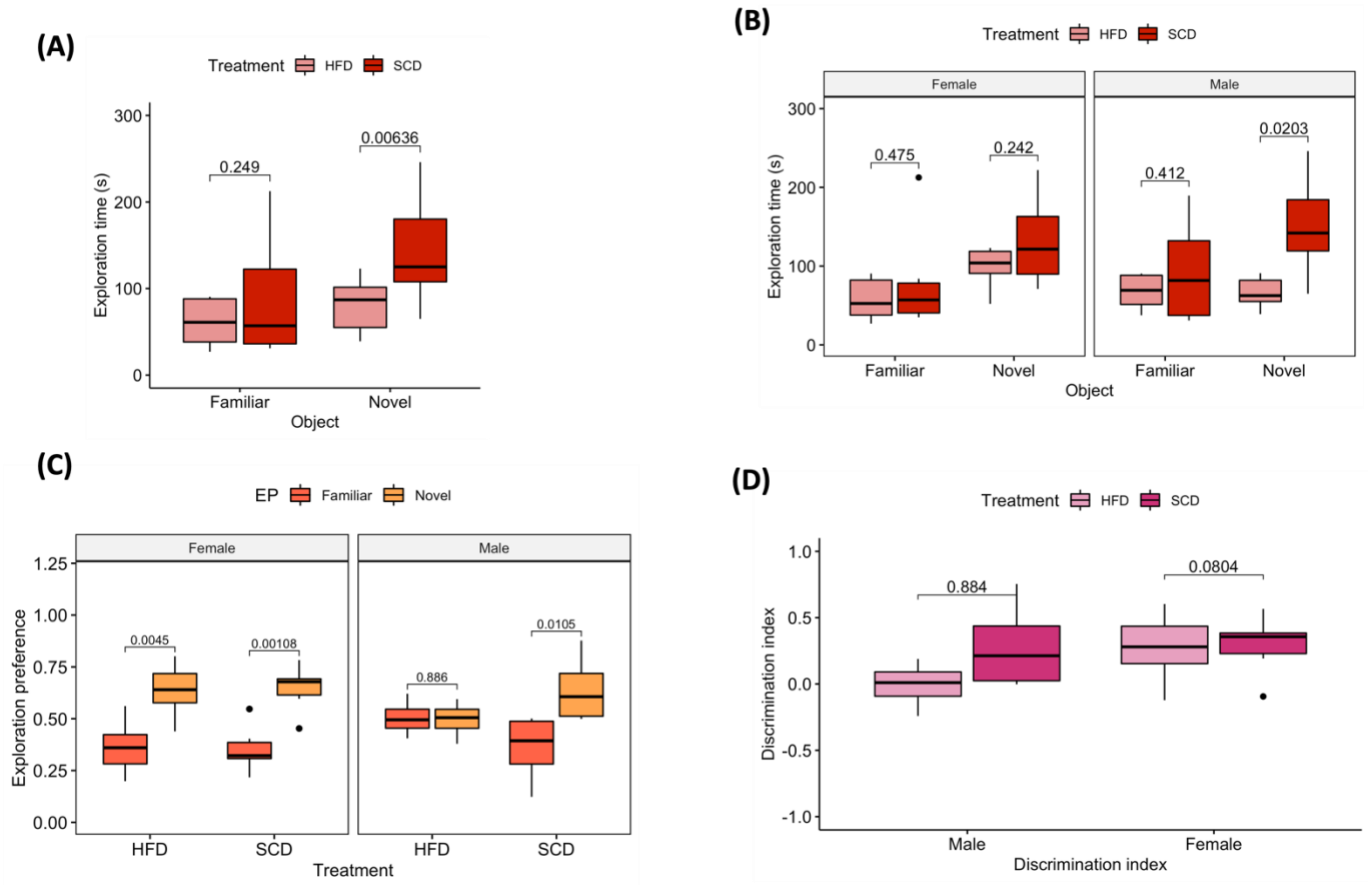

Figure 4. Novel object recognition test by male and female mice fed with HFD or control SCD. A) Exploration time in seconds of the familiar object and novel object by treatment. B) Exploration time in seconds of the familiar object and novel object by sex. C) Exploration preference of the familiar object and novel object by sex. D) Discrimination index (DI) by sex. SCD (in dark color) and HFD (in light color). Numbers above represent *p*-values obtained from t-test analysis between experimental groups.

Discussion

After 16 weeks in which mice were fed with a HFD, the obesity model was successfully achieved, regardless of sex. These results coincide with previous studies using diets with similar percentage of fat (60%) [39–44]. Similarly, visceral fat increased significantly regardless of gender, this is due to an increase in their calorie intake with HFD. This excess in calories is accumulated in the form of adipose tissue, indicating hyperplasia or hypertrophy of the adipose tissue, with an increase in the number of adipocytes or an increase in their size, respectively[45]. Surprisingly, the increase in body weight was highly notorious in males compared with females. Our results were similar compared with other studies using an alike obesity model in mice, where they found a higher increase in body weight in males than in females[46,47]. However, it also been reported that females are more susceptible to body weight gain than males[48,49]. We hypothesize that there is a correlation between body weight gaining and short-term memory, and/or a protective hormonal factor in females[50].

On the other hand, metabolic markers (glucose, triglycerides, and cholesterol) were not affected throughout 16 weeks, since they did not exceed the normal range. We observed an increase in glucose in males relative to females over time, but not in the other metabolic markers. Perhaps, these markers could be altered if the treatment time were extended, as reported for males[51]. However, the objective of this work was to induce a pattern of obesity, which was confirmed with an increase of 15% to 20 g of body weight between the HFD and control groups[38].

We observed that 16 weeks of HFD consumption affected mice performance on NORT. The novel object exploration time was significantly shorter, exploration preference and discrimination index were also negatively affected in HFD-fed male mice, which in fact, gained more body weight than females. These results are similar to a previous study that used the same obesity model with an HFD of 60% fat and showed that HFD decreases short-term memory in the novel object recognition test in C57BL6 male mice[52]. To the best of our knowledge, there is no evidence that HFD affects females in the same way. In another study using 15 months old male C57BL6 mice fed during three months with HFD (60%) showed deficits in discriminating novel places and special learning and showed a decrease in the DI effect in NORT[53]. There is evidence that performance on NORT and similar tests of recognition memory involve hippocampal function[54]. A recent study showed that Wistar rats fed with a HFD of (59.28% energy from fat) over 12 weeks caused dysbiosis. This effect was associated with decreased dendritic spine density, elevated ionized calcium-binding adapter molecule 1^{+} cells, increased levels of hippocampal reactive oxygen species and apoptosis with cognitive decline[55]. In another mouse study, it was shown that in young mice (3-week-old), HFD suppresses relational memory flexibility, assessed after initial learning of simultaneous radial maze spatial discrimination, and decreased neurogenesis[51].

Taken together, our results suggest that HFD induced obesity in mice, and that male mice are more susceptible to HFD, leading to obesity. Short-term memory, i.e., exploration time is affected in males, but not in females. To the best of our knowledge, this is the first study in the literature to use female mice in NORT with HFD. These changes in behavior might correlate with weight gain and potentially with dysbiosis of the intestinal microbiota, through the gut-brain axis, and this can trigger the expression of proinflammatory cytokines, better known as neuroinflammation, which is closely related to disorders of the behavior in animals. However, in the next work, we will soon elucidate the gut microbiota composition by sequencing the 16s rRNA gene from the stool samples obtained throughout this experiment and measure markers of inflammation in plasma and the brain.

Methods

Animal model and experimental design. 12-week-old healthy C57BL6 mice of approximately 20 g in weight were obtained from the Instituto de Neurobiología, Universidad Nacional Autónoma de México. Mice were housed in a mouse room at the Instituto Potosino de Investigación Científica y Tecnológica.

A total of 24 mice (12 females and 12 males) were used and divided into four groups: control male (n=6), experimental male (n=6), control female (n=6) and experimental female (n= 6).The control group was fed with a standard control diet (SCD: *Labdiet* ® 5001, USA) and the experimental group was fed with a high-fat diet (HFD) which provided 60% of total energy from fat (which was made in the laboratory) and 5% sucrose in drinking water for 16 weeks, Table 2 shows the nutritional composition of both diets. Body weight, food and water consumption were measured three times per week. Metabolic markers (blood glucose, cholesterol, and triglycerides) were measured every 4 weeks, using one drop of blood for each metabolic marker (see blood sample collection). In addition, fecal samples were collected every four weeks for further analysis (see below). A behavioral test was also performed at the end of 16 weeks to measure short-term memory using NORT (see below).

All animals were maintained in agreement with the ethical recommendations of the Norma Oficial Mexicana de especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio (NOM 062-ZOO-1999). Mice were maintained with light/dark cycles (12h×12h) at a temperature of 20-25°C, relative humidity of 30-60%, and housed individually in a polycarbonate

cage with grids, with a lid with a HEPA filter. The mouse cage was conditioned with a sterile cob litter. The animals underwent a two-week adaptation period before starting the experiment and were fed a standard diet for rodents (*Labdiet* ® 5001, USA) and water ad libitum.

Blood sample collection. For blood sampling, mice were fasted for 12 hours. Punctures in the caudal tail vein of mice and three drops of blood were collected. To measure the metabolic parameters, the AccuTrend Plus® meter was used with its respective reactive strips.

The novel object recognition test. One day after the 16-week period, each mouse was placed in a chamber (40 × 40 × 40 cm). The test consisted of three phases: habituation, familiarization, and test phase. In the habituation phase, each animal was allowed to freely explore the chamber in the absence of objects (5 minutes). The animal was then removed from the chamber and placed in its holding cage. During the familiarization phase, a single animal was placed in the chamber containing three identical sample objects and was allowed to freely explore for ten minutes. In the test phase, one of the sample objects was exchanged for a new object and the animal was returned to the chamber to explore the three objects, two were identical and the third was changed, the mouse was allowed to freely explore for another ten minutes. The duration of behavioral exploration exhibited by mice was determined with a stopwatch and exploration preference was calculated using the formula: (time exploring familiar object)/(time exploring new object + time exploring familiar object) or (time exploring familiar object)/(exploring novel object + time exploring familiar object). A discrimination index was calculated using the following formula: (time exploring novel object − time exploring familiar object)/(time exploring novel object + time exploring familiar object) [56].

Statics analysis. To analyze the metabolic markers (weight, glucose, triglycerides, and cholesterol), the *lm()* and *anova()* functions of the R programming language was used to perform ANOVA tests, the post hoc analysis *(T.test)* were performed by the *t_test()* function from rstatix package. Core Team (2021) test. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org

Animal sacrifice. At week 16 of feeding with HFD, mice received an intraperitoneal injection of sodium pentobarbital (30 mg/kg), and once the loss of sensitivity was verified, the heart was exposed by opening the cavity chest cavity and perfused with 25 mL of 0.9% sodium chloride. One group was used for dissection of tissues of interest and another group was perfused with 35 mL of 4% paraformaldehyde in 0.1 mM phosphate buffer for subsequent immunohistochemical assays.

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Supporting information

Treatment	Gender	Time (in weeks)	variable	n	mean	sd
HFD	Female	0	Weight	6	20.000	1.673
HFD	Male	0	Weight	6	22.833	2.639
HFD	Female	4	Weight	6	22.667	2.422
HFD	Male	4	Weight	6	26.333	3.445
HFD	Female	8	Weight	6	25.000	2.422
HFD	Male	8	Weight	6	30.833	5.307
HFD	Female	12	Weight	6	29.000	7.925

HFD	Male	12	Weight	6	35.000	8.672
HFD	Female	16	Weight	6	31.333	9.459
HFD	Male	16	Weight	6	38.333	11.961
SCD	Female	0	Weight	6	17.500	3.564
SCD	Male	0	Weight	6	22.500	1.378
SCD	Female	4	Weight	6	19.000	1.673
SCD	Male	4	Weight	6	23.500	1.761
SCD	Female	8	Weight	6	20.000	2.000
SCD	Male	8	Weight	6	24.333	1.966
SCD	Female	12	Weight	6	20.833	0.722
SCD	Male	12	Weight	6	25.500	2.074
SCD	Female	16	Weight	6	20.500	1.378
SCD	Male	16	Weight	6	24.833	2.137
HFD	Female	0	Glucose	6	196.667	35.613
HFD	Male	0	Glucose	6	180.167	17.680
HFD	Female	4	Glucose	6	197.667	29.063
HFD	Male	4	Glucose	6	211.000	26.556
HFD	Female	8	Glucose	6	194.000	27.452
HFD	Male	8	Glucose	6	233.333	52.053
HFD	Female	12	Glucose	6	191.667	23.441
HFD	Male	12	Glucose	6	241.833	31.676
HFD	Female	16	Glucose	6	178.500	27.790
HFD	Male	16	Glucose	6	235.167	39.005
SCD	Female	0	Glucose	6	176.333	53.444
SCD	Male	0	Glucose	6	160.667	44.523
SCD	Female	4	Glucose	6	177.000	17.065
SCD	Male	4	Glucose	6	214.333	26.546
SCD	Female	8	Glucose	6	162.667	11.793
SCD	Male	8	Glucose	6	216.000	50.323
SCD	Female	12	Glucose	6	176.167	29.233
SCD	Male	12	Glucose	6	199.000	29.086
SCD	Female	16	Glucose	6	183.167	29.034
SCD	Male	16	Glucose	6	199.167	31.154
HFD	Female	0	Triglycerides	6	75.333	5.086
HFD	Male	0	Triglycerides	6	104.667	38.370
HFD	Female	4	Triglycerides	6	77.167	2.401
HFD	Male	4	Triglycerides	6	89.333	10.652
HFD	Female	8	Triglycerides	6	95.167	16.916
HFD	Male	8	Triglycerides	6	103.667	25.633
HFD	Female	12	Triglycerides	6	97.167	16.302
HFD	Male	12	Triglycerides	6	99.500	20.345
HFD	Female	16	Triglycerides	6	101.333	19.086
HFD	Male	16	Triglycerides	6	116.667	11.147

SCD	Female	0	Triglycerides	6	93.667	11.483
SCD	Male	0	Triglycerides	6	100.333	13.852
SCD	Female	4	Triglycerides	6	87.167	14.034
SCD	Male	4	Triglycerides	6	73.167	13.167
SCD	Female	8	Triglycerides	6	82.833	7.548
SCD	Male	8	Triglycerides	6	90.667	8.066
SCD	Female	12	Triglycerides	6	86.667	16.367
SCD	Male	12	Triglycerides	6	79.333	8.066
SCD	Female	16	Triglycerides	6	87.167	6.585
SCD	Male	16	Triglycerides	6	95.000	25.908
HFD	Female	0	Cholesterol	6	160.667	2.422
HFD	Male	0	Cholesterol	6	159.000	6.229
HFD	Female	4	Cholesterol	6	160.667	7.174
HFD	Male	4	Cholesterol	6	164.000	7.874
HFD	Female	8	Cholesterol	6	159.833	5.307
HFD	Male	8	Cholesterol	6	162.667	3.670
HFD	Female	12	Cholesterol	6	164.000	4.290
HFD	Male	12	Cholesterol	6	161.667	8.524
HFD	Female	16	Cholesterol	6	158.833	4.665
HFD	Male	16	Cholesterol	6	157.667	5.203
SCD	Female	0	Cholesterol	6	158.333	4.761
SCD	Male	0	Cholesterol	6	159.500	5.431
SCD	Female	4	Cholesterol	6	159.500	11.309
SCD	Male	4	Cholesterol	6	153.167	25.988
SCD	Female	8	Cholesterol	6	160.333	4.412
SCD	Male	8	Cholesterol	6	162.333	7.174
SCD	Female	12	Cholesterol	6	157.000	5.441
SCD	Male	12	Cholesterol	6	158.667	5.164
SCD	Female	16	Cholesterol	6	154.500	4.506
SCD	Male	16	Cholesterol	6	153.667	3.386

Table 1. Means and standard deviation (SD) of body weight, glucose, triglycerides, and cholesterol of the experimental groups.

	SCD			HFD		
	Percentage (%)	**Grams (g)**	**Energy (Kcals)**	**Percentage**	**Grams (g)**	**Energy (Kcals)**
Protein	28.50	0.29	0.96	13.84	0.29	1.14
Fat	13.50	0.13	0.45	63.92	0.58	5.26
Carbohydrates	58.00	0.58	1.95	22.24	0.46	1.83
Total	100.00	1.00	3.36	100.00	1.33	8.24

Table 2. Nutritional composition of SCD and HDF.