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Sex-specific variations in spatial reference memory acquisition: Insights from a comprehensive behavioral test battery in C57BL/6JRj mice

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ABSTRACT

Sex differences in declarative memory are described in humans, revealing a female or a male advantage depending on the task. Specifically, spatial memory (i.e., spatial navigation) is typically most efficient in men. This sexual dimorphism has been replicated in male rats but not clearly in mice. In this study, sex differences in spatial memory were assessed in thirty-six C57BL/6 J mice (Janvier Labs; i.e., C57BL/6JRj mice), a widely used mouse substrain. Both male and female mice (12 weeks-old) were subjected to standard behavioral paradigms: the elevated plus maze, the open field test, the novel object and place tests, the forced swimming test, and the water maze test for spatial navigation. Across assessment, no sex differences were found in measures of locomotor activity, emotional and behavioral responses, and object and place recognition memories. In the water maze, male mice were faster in learning the platform location in the reference memory training and used more spatial strategies during the first training days. However, both sexes reached a similar asymptotic performance and performed similarly in the probe trial for long-term memory consolidation. No sex differences were found in the cued training, platform inversion sessions, or spatial working memory sessions. Hippocampal expression of the brain-derived neurotrophic factor was similar in both sexes, either in basal conditions or after performing the behavioral training battery. Importantly, female mice were not more variable than males in any measure analyzed. This outcome encourages the investigation of sex differences in animal models and the usefulness of including female mice in behavioral research.

1. Introduction

Declarative memory comprises unique personal experiences -episodic memory-, factual knowledge -semantic memory- and memory that requires the spatial integration of stimuli -spatial and contextual memory- [1]. In the field of cognitive neuroscience, declarative memory has received wide attention regarding the study of sex differences in humans, mostly revealing a small but significant female advantage in episodic and semantic memories [2,3]. A recent meta-analysis [2] compiling studies with 1,233,921 participants concluded that females outperform males in long-term declarative memory but there are differences regarding the material to be remembered. In this way, females seem more proficient in memory that involves verbal information -e.g. words, sentences, images that could be named,…- faces, colors, tastes or odors; while males have an advantage in memory for abstract concepts and in spatial memory tasks including spatial navigation. This behavioral outcome in each sex may be accompanied by different neurobiological correlates in key regions supporting declarative memory, such as the hippocampus [1]. The most recent data does not clearly show the volume and overall structure of the human hippocampus to be sexually dimorphic -once corrected for overall brain size- [4,5], but the hippocampal functional activation and connectivity when performing a memory task seem to vary for each sex [6].

Also anxiety and depressive disorders exhibit a higher prevalence

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among women compared to men [7,8]. While psychological and cultural factors may contribute to these sex and gender differences [9], there is compelling evidence suggesting the involvement of biological factors as well [10]. For instance, women experiencing depression often show an earlier onset, reduced quality of life, and an increased likelihood of comorbidity with anxiety disorders when compared to men with depression [11,12]. Consequently, there is an ongoing need to investigate anxiety and depression-related behaviors in female mice [13].

Animal research is a valuable tool to unveil different neurobiological mechanisms supporting cognitive functions between sexes without the ethical and methodological limitations that constrain human research. Nowadays, governments are currently investing efforts to urge researchers to investigate sex differences and to incorporate female animals in biomedical studies [14]. Unfortunately, a strong male bias still persists [15] since female animals have been traditionally severely underutilized in neurobehavioral research compared to males [16,17]. This circumstance may be explained by cultural issues but also by the historical belief that female laboratory rodents are intrinsically more 'variable' than males due to hormonal changes across the estrous cycle; so data obtained from females were assumed as less generalizable or as requiring larger sample sizes for statistical significance [16–18]. Accordingly, experiments that used female mice or rats were frequently demanded to perform daily tracking of their 4-day estrous cycle through vaginal cytology, which was viewed as both costly and time-consuming. In recent years, key reviews and meta-analyses have concluded that, for many neurobiological and behavioral traits, data obtained from female rats and mice tested at random estrous phases showed no more variability than data obtained from males [17,19–21]. In other words, despite well-established sex differences, most of these variables do not seem to fluctuate across the female estrous cycle strongly; and the inclusion of females in biomedical research is currently encouraged [16,17,20,21].

In the absence of verbal memory in animals, declarative memory in rodents is frequently investigated by spatial navigation tasks -a cognitive function in humans in which males usually outperform females [2,22]-; with the Morris water maze being a 'gold-standard' paradigm in behavioral neuroscience. Notably, a large retrospective study with ~2650 mice from each sex, reported that female mice do not perform more variable than males in this task [20]. Regarding sex differences, a meta-analysis concluded that -after controlling for relevant variables such as the age of the animals and the number of training trials- studies in rats tested in the water maze and other spatial tasks revealed a male advantage [23], which mirrors the outcome found in humans. However, the same meta-analysis concluded that studies examining sex differences in the water maze are still insufficient, heterogeneous and inconclusive for mice [23]. Indeed, results of different mouse strains tested in standard water maze protocols have either favored males [– 129/SvJ mice- [24]; -C57BL/6NIA mice (specifically at 17 months-old)- [25]; -C57BL/6 J mice- [26]]; favored females [-ICR mice- [27], -NMRI mice- [28]] or did not report sex differences (-C57BL/6 J mice- [29,30]; –129S2/SvHsd, C57BL/6JOlaHsd, FVB/NHsd; 129S2/SvHsd×C57BL/6JOlaHsd and 129S2/SvHsd×FVB/NHsd mice- [31] -C57BL/6NIA mice (specifically at 5 and 25 months old)- [25]). A remarkably large study by Fritz et al., which mainly used mice from heterogeneous genetic backgrounds, concluded that males outperformed females in variables related to both the acquisition and retention (i.e. probe trial) of the platform location. However, while such effect was significant, it was negligible in its magnitude [20]. To explain these divergent results across studies, in addition to factors related to the behavioral protocols, it should be considered that a notable variability is described among mouse strains and even among mouse substrains, that show both physiological and behavioral differences [32,33]. Importantly, the mouse strain and substrain are relevant factors that determine the existence, magnitude, and even the direction of sex differences in baseline behavior [31,34,35].

Considering the importance of bridging the gap between sexes in animal behavioral neuroscience, and the variability of current data, the main aim of this manuscript is to study sex differences in spatial memory in C57BL/6 J mice. The C57BL/6 J is a widely used laboratory inbred mouse substrain considered representative in many research fields since it was the first line in which the mouse genome was sequenced [33]. Specifically, we used C57BL/6 J mice of both sexes obtained from a European vendor (C57BL/6JRj mice) that were assessed in a behavioral test battery for exploratory, emotional and cognitive-related variables, with a particular interest in spatial memory performance in the water maze. Additionally, we evaluated hippocampal levels of brain-derived neurotrophic factor (BDNF) in both sexes; either in basal conditions or after performing the behavioral training. This neurotrophin has been proposed as a biomarker of brain plasticity and cognitive performance in both animals and humans [36], but data comparing hippocampal BDNF levels between sexes in mice are still scarce.

2. Methods

2.1. Animals

Thirty-six young-adult C57BL/6JRj mice – 18 mice of each sex-, were acquired from Janvier (Le GenestStIsle, France) and arrived at the animal facility when they were 10 weeks of age. Female mice were sexually-naïve. After one week of acclimation, mice were individually housed in standard laboratory cages with nesting material and were handled twice a week (5 min/day) for five weeks. The animals were maintained on a 12 h light/dark cycle (lights on at 8:00 a.m.) with water and food provided ad libitum, and cages were changed once every 2 weeks. It is essential to consider the timing of experiments in light of the circadian rhythm to ensure accurate and meaningful results. Female mice were not tracked for estrus cyclicity.

Procedures were performed according to the European and Spanish regulations for animal research (Directive 2010/63/UE, Real Decreto 53/20,130 and Ley 32/2007) and were approved by the research ethics committees of the University of Málaga (code: 104–2021-A) and Junta de Andalucía (code: 3/11/2021/170).

2.2. Behavioral testing

The behavioral assessment started five weeks after the separation of mice in individual cages. Mice were carried to a noise-isolated room (illuminated 120 lux) at 9:00 a.m. and were habituated for at least 20 min before starting the behavioral testing. A solution of 30% ethanol was used to clean the maze arena and eliminate odor cues. Sessions were recorded and spatiotemporal parameters were analyzed with the software Ethovision XT.17. (Noldus, Wageningen, The Netherlands). As the sex of experimenters can influence mouse behavior [37], we disclose that a female experimenter conducted the behavioral assessments. Observational scorings were carried out by a trained observer who was blind to the mice's sex and had no previous assumptions about the study's outcome.

Assessment of exploration, emotional responses and cognitive performance was carried out based on our previously published protocols that were performed in adult C57BL/6JRj male mice only [38,39]. The tasks were conducted in a specific order of increasing invasiveness (Fig. 1**A**). Unconditioned anxiety-like behavior and spontaneous exploration tasks were performed early to minimize familiarity-induced reductions in exploration. This tasks included Elevated Plus Maze (EPM) and Open Field Test (OFT). The study also included Novel Object Recognition (NOR) and Novel Place Recognition (NPR) tests that require little training and assess mice's natural tendency to explore novelty. The Forced Swim Test (FST) was performed later to minimize the potential acute stress influence on consecutive tasks. After a resting period, water maze training was conducted, which is more invasive and involves physical exercise, learning, and hippocampal changes.

The behavioral schedule was structured as shown in Fig. 1**A**:

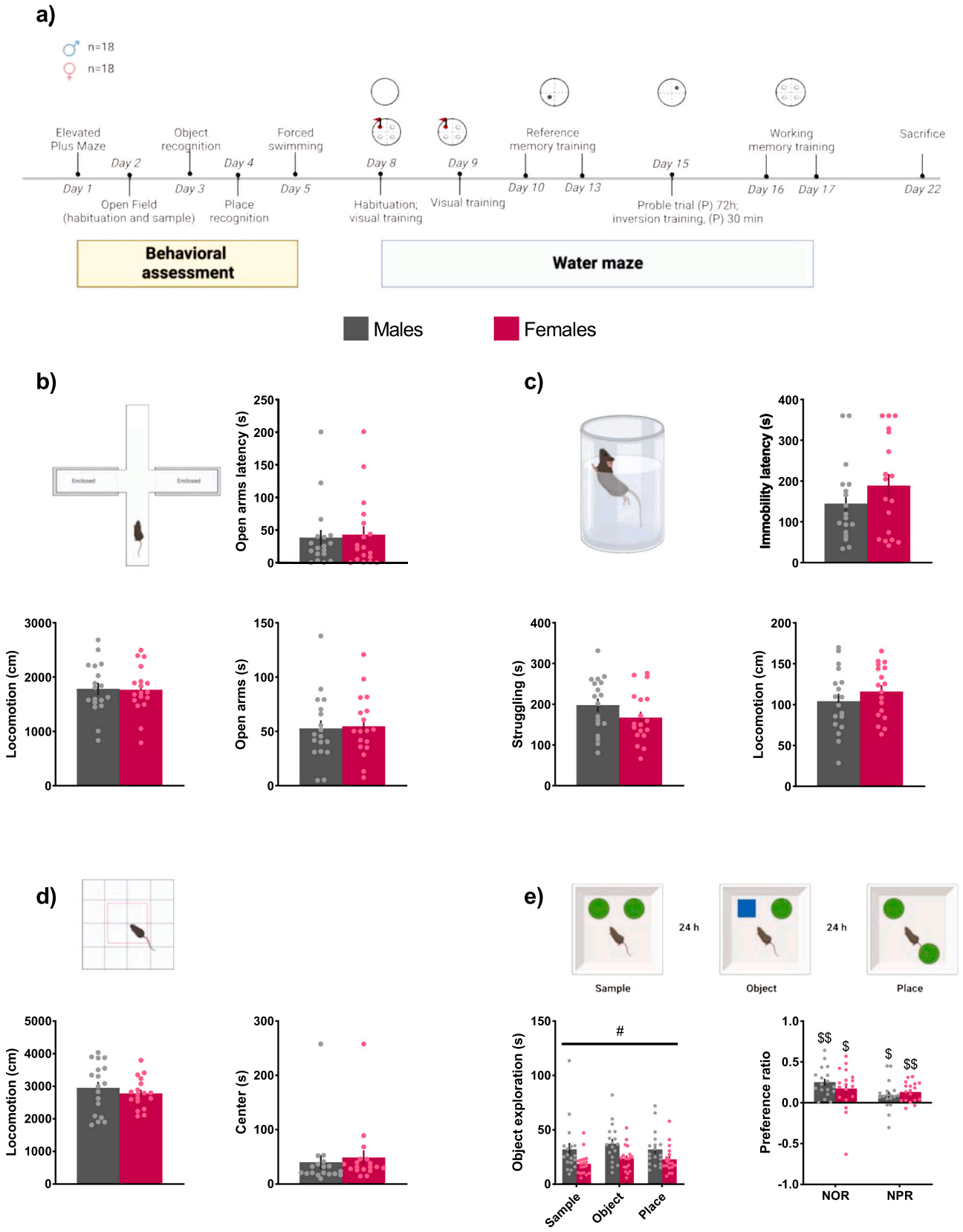

Fig. 1. (a) Behavioral testing protocol. Results from the elevated plus maze (b), the forced swimming test (c), the open field habituation (d), and the object and place memories (e) did not reveal differences between sexes, except for a reduced time of object exploration in female mice (e). Student's t-test: difference versus zero: $p < 0.05; $$p < 0.001; Analyses of variance (ANOVA) effect for sex—reported in the main text: #p < 0.05. Data are expressed as mean ± SEM. Created with BioRender.com.

•***Elevated plus maze*** **(EPM)** (Day 1). The plus-shaped (+) apparatus was elevated 47 cm above the floor and consisted of two unprotected open arms and two enclosed arms (measuring 29.5 ×5 cm each) connected by a central platform (5 ×5 cm). The mouse was released in the center platform and allowed to explore freely for 6 min. Locomotion (cm) and time spent (s) in the open arms, as well as the latency to enter an open arm (s) were analyzed.

•***Open field test*** **(OFT)** (Day 2). On the second day, mice were placed in the corner of an empty open field (40 ×40, 40 cm high) and allowed to explore for 5 min (habituation session). Total locomotion (cm) and time spent (s) in the center zone (comprised of an imaginary central square of 20 ×20 cm) were evaluated. Total time performing rearing (the mouse stands on its hind legs while its front legs may or may not be supported by the walls), risk assessment (the mouse stretches its head and front legs forward and then returns to its initial posture without locomotion of its hind legs) and grooming (the mouse licks or tidies up its fur, whiskers, ears, paws or tail) were analyzed.

•***Novel object recognition*** **(NOR)** ***and novel place recognition*** **(NPR)** ***memories*** (Days 2–4). Sixty minutes after the OFT, mice were re-exposed to the apparatus, including two identical copies of an object ('familiar' object) placed near two adjacent corners (sample session) and allowed to explore for 10 min. On Day 3, mice were left to explore for 10 min an identical copy of the familiar object and a 'novel' unknown object, located in the previous positions (NOR test). Finally, on Day 4, the open field was furnished with two identical copies of the familiar object, where one of them was placed in its habitual location (i.e. 'stationary'), while the other was 'displaced' to the opposite corner (NPR test); and the mouse explored for 10 min. Total seconds of object exploration (defined as nose or paw contact with the object or pointing its nose towards the object at a distance of 1–2 cm away) was analyzed as per prior research (Mañas-Padilla et al., 2022) and the 'object memory ratio' [(time exploring the novel object-time exploring the familiar object)/ total time exploring both objects] and the 'place memory ratio' [(time exploring the displaced object – time exploring the stationary object)/ total time exploring both objects] were calculated to gauge object and place memory, respectively.

•***Forced swimming test*** **(FST)** (Day 5). Mice were placed in a transparent cylinder (10 cm diameter, 27 cm height) containing water (22 ± 1 °C) to a depth of 15 cm for 6 min. Three different behaviors, namely 'immobility' (minimal movements to keep the head above water), 'struggling' (vigorous attempts to climb the cylinder walls and break the water surface using the forelimbs), and 'swimming' were observed and recorded.

•***Water maze*** (Days 8–17). The circular pool of the water maze was filled with opaque water (made of non-toxic white paint) at a temperature of 24ºC ± 1ºC, and was divided into four equal imaginary quadrants. Mice were released from one of four possible starting positions in each quadrant's peripheral region (north -N-, west -W-, east -E-, and south -S-). The room was equipped with distal extra-maze cues to facilitate spatial orientation, such as visible black cardboard in a distinctive geometric shape on each wall and various furniture elements. Latency (s) to reach the platform and total path length (cm) were assessed during the training session as learning measures.

The water maze training was as follows:

- Habituation (Day 8). Mice were habituated to swimming for a 1 min session, starting from the S position. The total distance swum (s), time spent in each quadrant and time spent in the peripheral area (defined as an outer zone 24 cm in from the walls), were analyzed.

-Visible platform training (Days 8–9). In mice, it is advantageous to administer this procedure at the beginning of training, for them to learn that the platform is the only escape route [40]. Forty-five min after habituation, a white goal platform (11 cm diameter) with a black polystyrene tube standing vertically was placed in the center of one quadrant, slightly above the water level. The mice received four daily training sessions (with an inter-trial interval –ITI– of 30 min), during which the platform position was changed across all quadrants, and the starting positions were alternated. Mice were removed from the pool when they reached the visible platform, and if the platform was not reached within 1 min, the experimenter guided the mouse to it. This training was repeated on Day 9. Path length (cm), velocity (cm/s), and latency (s) to reach the platform were analyzed to assess whether mice exhibited normal behavior, including factors such as eyesight, motoric ability, and understanding of task rules. Additionally, this analysis aimed to gauge their motivation, specifically their willingness to escape the water, when performing the water maze task.

-Reference memory training (Days 10–13). The platform was placed permanently in the target quadrant, submerged under 1 cm of water. Spatial memory training was conducted for four consecutive days (Days 10–13), with six training sessions each day (IT: 30 min). In each session, mice were released into the pool from one of the four starting positions (N, W, E, S). The mice were allowed to stay on the hidden platform for 5 s after finding it, and if not found within 1 min, the experimenter guided the mouse to it, allowing the mouse to remain on the platform for 10 s. To assess learning in the Morris water maze, we examined the mice's search strategies during spatial memory training, following Brody and Holtzman's (2006) method. For clarity, we categorized strategies into two groups. The first, termed spatial strategies, included 'direct' (swimming straight to the platform), 'indirect' (a single turn towards the platform), and 'focal correct' (directly swimming to and intensely searching the platform quadrant) approaches. The second, termed non-spatial strategies, encompassed 'scanning' (searching the tank without spatial preference), 'random' (exploring the entire tank without preference), and 'focal incorrect' (intensely searching a small platform-free area) approaches. Additionally, other behaviors related to searching for the platform were included, such as repetitive looping patterns like 'chaining' (circular path >15 cm from the pool's wall), 'peripheral looping' (persistently swimming around the pool's outer 15 cm), and 'circling' (tight circles). Motionless mice were excluded from the study.

-Probe trials (Day 15). The platform was removed from the maze and mice were released from the S position and given 1 min to swim. Their performance was measured by evaluating the total time (s) spent swimming inside the target and opposite quadrants, the latency (s) to reach the circular region where the platform was previously located, and the number of crossings made to the imaginary platform. Two probe trials were conducted on Day 15. The first one was performed early in the day, 72 h after the last spatial training session, to assess long-term retention of spatial memory. The second one was conducted 30 min after the last training session for cognitive flexibility, to evaluate short-term memory acquisition of the new platform location.

-Training with platform inversion (Day 15). Forty-five minutes after the first probe trial, the platform was hidden in the center of quadrant E, opposite to the target quadrant employed during spatial training. Six training sessions were conducted as outlined earlier to assess cognitive flexibility.

-Working memory training (Days 16–17). A delayed matching-to-sample protocol consisted of four training sessions (ITI: 30 min); each composed of a sample and a test trial with a 60-second temporal gap between them. In the sample trial, the mouse had to learn the location of the platform, which could be hidden in the center of any of the four quadrants of the pool. In the test trial, the mouse was released from the same starting position as the sample trial and had to recall the platform's location. Both the sample and test trials had a maximum duration of 1 min. The measures during the sample and test trials were averaged separately on a daily basis for graphical representation and statistical analysis.

2.3. Brain samples collection

Mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg·kg−1 BW) and sacrificed on Day 22, three days after the end of the behavioral assessment. Five minutes after administering the

anesthetic, mice were immediately killed by decapitation. Brain samples were immediately extracted and frozen at − 80 °C for late analysis. The left hemisphere of the hippocampus was dissected from frozen brain samples by using the Paxinos and Watson's mouse brain atlas [41]. The hippocampus (17–20 mg per sample) and prefrontal cortex samples (18–21 mg per sample) were homogenized in 1 ml of cold radioimmunoprecipitation assay buffer lysis buffer (RIPA); 50-mM Tris–HCl pH 7.4, 150-mM NaCl, 0.5% Sodium Deoxycholate, 1-mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton, 0.1% SDS, 1-mM Na3VO4, 1-mM NaF, complemented with a phosphatase (Phosphatase Inhibitor Cocktail Set III, Millipore, 524527) and a protease (cOmplete™ Protease Inhibitor Cocktail, Roche, 11836145001) inhibitor cocktail. After an incubation of 2 h at 4 °C, the suspension was centrifugated at 12,000 rpm for 15 min at 4 °C. The protein extracts (obtained in the previous supernatant) were diluted 1:1 in loading buffer (Ditiotreitol [DTT] 2X) and heated for 5 min at 99 °C.

2.4. Western blot

Protein expression for BDNF was measured by western blot following methods from our laboratory [42]. The tissue protein (10–15 μg) was subjected to electrophoresis on 4–12% Criterion XT Precast Bis-Tris gels (Bio-Rad, California, USA) for 30 min at 80 V, followed by 2 h at 150 V. The proteins were transferred onto a 0.2-μm nitrocellulose membrane (Bio-Rad, USA) using wet transfer equipment (Bio-Rad, USA) for 1 h at 80 V. A Ponceau Red staining (10x diluted to 1x in H_2O) has been used to visualize the proteins. Then, the membrane was washed with TBST 1X Tween 20 (150-mM NaCl, 10-mM Tris-HCl, 0.1% Tween 20, pH 7.6) until it disappears and becomes totally clean, and blocked with 2% bovine serum albumin-Tris buffered saline Tween 20 (BSA-TBST1X) for 1 h on a shaker platform at room temperature. The membrane was then incubated with the primary rabbit anti-BDNF antibody (1:250, AB1534SP, Sigma-Aldrich) diluted in 2% BSA-TBST1X overnight at 4 °C. The following day, the membrane was washed three times for 10 min with TBST 1X and incubated with a Horseradish Peroxidase conjugated secondary antibody (Goat anti-rabbit IgG, W4011, Promega, USA) diluted 1:10,000 in 2% BSA-TBST 1X for 1 h at room temperature on a shaker platform. After washing the membrane, it was exposed to a chemiluminescent reagent (Santa Cruz Biotechnology Inc., USA) for 5 min. If required, stripping/reproving steps were used. The membrane-bound protein was visualized by chemiluminescence (ChemiDoc Imaging System, Bio-Rad, USA) and bands were quantified using ImageJ software (densitometric analysis http://imagej.nih.gov/ij). Normalization was performed using a reference protein, the γ-adaptin (diluted 1:2000, 610385, BD Biosciences), which was present on the same membrane. The obtained results were expressed as the ratio between the total protein expression and γ-adaptin, as described by Bass et al. in 2017.

2.5. Statistical analysis

Statistical analyses were performed either by Student's *t*-tests for independent or dependent samples (when appropriate) or by analyses of variance (ANOVA) followed by *posthoc* Fisher's least significant difference (LSD) when required. In those spatial memory measures where the 'sex' effect resulted significantly, we aimed to evaluate the effect's size and the variability of the data obtained from each sex. The size of the effect was computed by Cohen's d (CId) at a 95% confidence interval using an online resource [https://www.psychometrica.de/effect_size.html] (Lenhard & Lenhard, 2016). Accordingly, effect sizes were classified as negligible ($0 \leq |d| < 0.2$), small ($0.2 \leq |d| < 0.5$), intermediate ($0.5 \leq |d| < 0.8$) or large ($0.8 \leq |d| \leq 1$). The variability was calculated using the coefficient of variance test [43]. To perform this test, we followed the instructions of this website (https://real-statistics.com/students-t-distribution/coefficient-of-variation-testing/). In the platform searching strategies analysis, the percentage of spatial strategies used by the animals was calculated and analyzed by Fisher's exact test.

3. Results

3.1. Male and female mice did not differ in locomotion and emotional behavior

There were no sex differences regarding exploratory and emotional variables analyzed in the EPM [Student's t-test: locomotion: $t(34) = 0.113$, $p = 0.910$; time in open arm: $t(34) = -0.203$, $p = 0.841$; open arm latency: $t(34) = -0.262$, $p = 0.800$; Fig. 1**B**], the FST [total immobility: $t(34) = -0.958$; $p = 0.345$; latency to first immobility: $t(34) = -1202$, $p = 0.238$; struggling: $t(34) = 1.402$, $p = 0.170$; Fig. 1**C**] and the OFT -habituation session- [locomotion: $t(34) = 0.805$, $p = 0.426$; time in center: $t(34) = -0.473$, $p = 0.639$; Fig. 1**D**]. Furthermore, during the OFT, there were no sex differences regarding to the total time performing behavioral parameters such as rearing [Student's test: $t(34) = 1.901$, $p = 0.065$; males: 41.344 ± 2.879, females: 34.494 ± 2.164 (mean ± SEM)], grooming [$t(34) = 0.567$, $p = 0.574$; males: 7.167 ± 0.763, females: 6.661 ± 0.747] and risk assessment [$t(34) = 1.217$, $p = 0.232$; males: 0.706 ± 0.271, females: 0.328 ± 0.151].

3.2. Object and place recognition tasks revealed reduced object exploration for females, but no differences in memory-related measures

During the sample and test sessions of the object recognition task, female mice explored objects for less time than males [repeated measures ANOVA' sex x session': effect for 'sex': $F(1, 34) = 6.937$, $p = 0.013$; 'session': $F(2, 68) = 2.226$, $p = 0.116$; 'sex x session': $F(2, 68) = 0.524$, $p = 0.595$; Fig. 1**B**]. Nevertheless, they showed similar locomotion [effect for 'sex': $F(1, 34) = 2.738$, $p = 0.107$; 'session': $F(2, 68) = 10.506$, $p < 0.001$; $F(2, 68) = 0.348$, $p = 0.707$] and time spent in the center [effect for 'sex': $F(1, 34) = 0.462$, $p = 0.501$; 'session': $F(2, 68) = 1.166$, $p = 0.318$; 'sex x session': $F(2, 68) = 0.130$, $p = 0.879$] across these trials (data not shown).

Regarding memory-related measures, both sexes showed a similar preference ratio for the novel object [Student's t-test: $t(34) = 1.013$, $p = 0.318$] and the novel place [Student's t-test: $t(34) = -0.758$, $p = 0.454$; Fig. 1**B**]. All preference ratios were significantly greater than zero [sample Student's t-tests: object memory ratio vs zero: males: $t(17) = 5.938$; $p < 0.001$; females: $t(17) = 2.737$; $p = 0.014$; place memory ratio vs zero: males: $t(17) = 2.229$; $p = 0.040$; females: $t(17) = 4.703$; $p < 0.001$; Fig. 1**B**], indicating that none of the groups performed by chance.

3.3. Female mice showed delayed learning of the platform location in the water maze

3.3.1. Habituation and visible platform training

In the water maze, female and male mice were similar in the sessions previous to memory training. During the habituation session, both groups of mice showed similar swimming distance [Student's t test: $t(34) = -1.147$, $p = 0.259$], spent the same time exploring the maze's periphery [$t(34) = -0.808$, $p = 0.435$] and did not differ on the time spent on each quadrant [repeated measures ANOVA' sex x session': effect for 'sex': $F(1, 34) = 0.009$, $p = 0.923$; 'session': $F(3, 102) = 1.753$, $p = 0.161$; 'sex x session': $F(3, 102) = 1.866$, $p = 0.140$; Fig. 2**A**]. In the visible platform training sessions, both sexes similarly learned the task across days [repeated measures ANOVA' sex x session': effect for 'sex': $F(1, 34) = 2.012$, $p = 0.165$; 'session': $F(7, 238) = 15.585$, $p < 0.001$; 'sex x session': $F(7, 238) = 0.864$, $p = 0.536$; Fig. 2**B**].

3.3.2. Spatial reference memory acquisition

In the reference memory acquisition sessions, female mice displayed

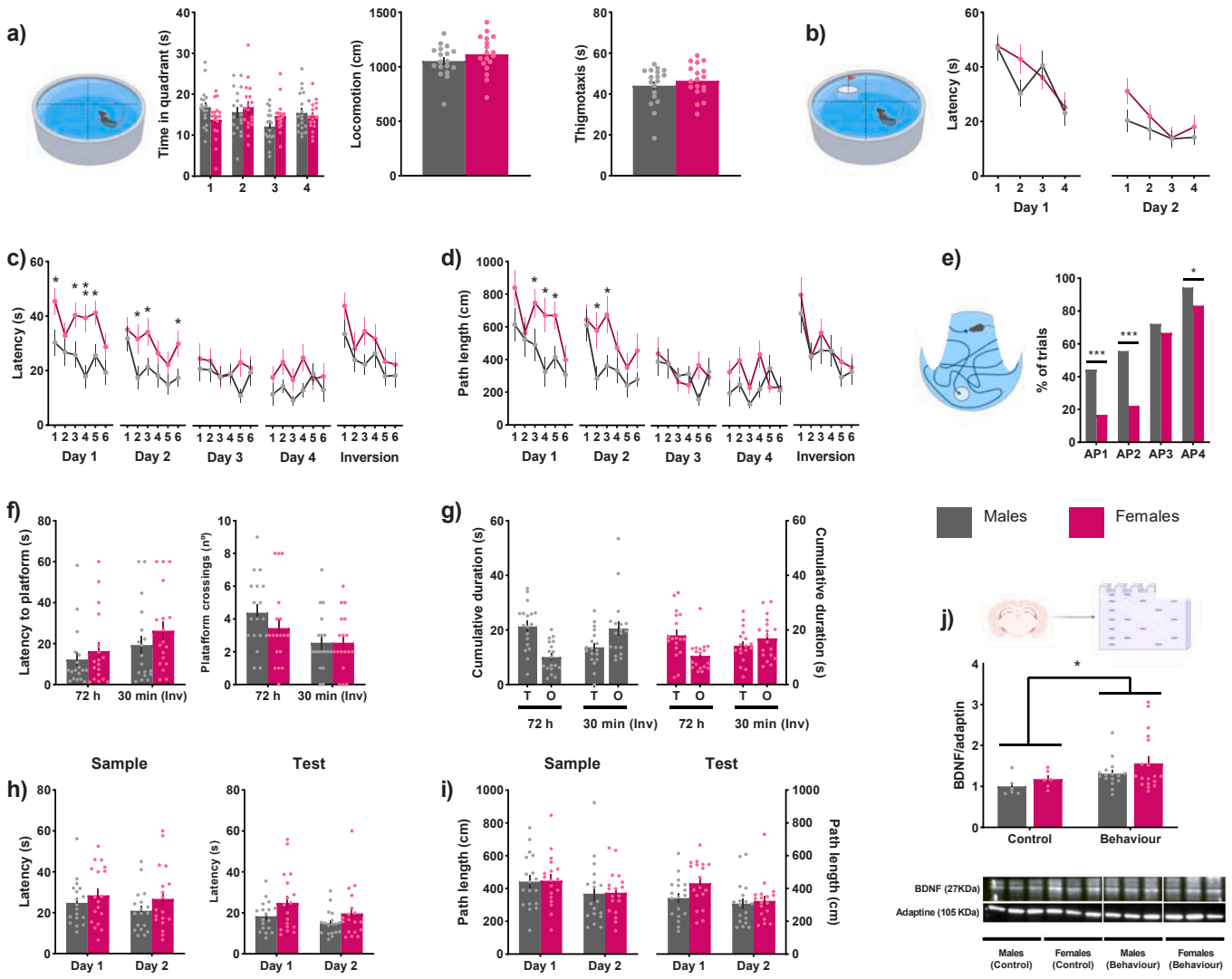

Fig. 2. Water maze results revealed delayed spatial reference memory acquisition in female mice. (a) Habituation session. (b) Training with the visible platform. (c, d) Reference memory acquisition and training with new platform location (i.e. inversion) showed that male mice outperformed females specifically at the initial training sessions, as well as they used more spatial strategies than females (e). (f, g) No sex differences were found in measures in the probe trials for long-term memory retention (72 h) and short-term acquisition of the new platform location (30 min; inversion) and in spatial working memory (h, i). Hippocampal BDNF levels were also similar between sexes (j). Post hoc least significant difference (LSD): difference between sexes: *p < 0.05; * *p < 0.001; difference between quadrants: $p < 0.05; $$p < 0.001. Data are expressed as mean ± SEM. Created with BioRender.com.

a delay in learning the hidden platform location, showing more latency to reach the platform [repeated measures ANOVA' sex x session': effect for 'sex': F(1, 34) = 5.389, p = 0.026; 'session': F(23, 782) = 6.783, p < 0.001; 'sex x session': F(23, 782) = 1.344, p = 0.130] and more distance swum -i.e. path length- [effect for 'sex': F(1, 34) = 6.239, p = 0.018; 'session': F(23, 782) = 7.190, p < 0.001; 'sex x session': F (23, 782) = 1.428, p = 0.088]. In this case, a post hoc analysis was performed for the 'sex x session' interaction. Although the p-value of 0.088 is not statistically significant, it is noteworthy as it was accompanied by a significant 'sex' effect (p = 0.018). The post hoc analysis was conducted to provide valuable conceptual information in comparing the sexes.

Because the 'sex x session' effect showed a tendency to signification, we performed a LSD posthoc analysis which suggested that differences between sexes were more evident during the first acquisition sessions (i.e. training days 1 and 2) (LSD post hoc analysis is shown in Fig. 2**C-D**). Swimming velocity (cm/s) was not significantly influenced by sex -data not shown- [effect for 'sex': F(1, 34) = 0.010, p = 0.923; 'session': F(23, 782) = 3.750, p < 0.001; 'sex x session': F(23, 782) = 1.466, p = 0.073]; which indicates that velocity could not account for results found in other measures.

Additional analysis (Table 1) confirmed both significant and large size effects for differences between sexes on training Day 1 (for latency to the platform and path length) and on training Day 2 (for path length) but not for training Days 3 and 4. Therefore, both sexes showed similar learning by the end of training. We also evaluated if the variability between groups was different using the coefficient of variance test (Table 1). Results showed behavioral similarities in males and females, showing equal variability.

3.3.3. *Long-term memory retention*

A probe trial performed 72 h after the last reference memory session did not reveal sex differences in the long-term retention of spatial memory. Sexes were similar in the latency to reach the previous platform location [t(34) = - 0.762, p = 0.452] and in the number of crossings to this location [t(34) = 1.229, p = 0.228] (Fig. 2**F**). Analyses of

Table 1
Spatial memory measures during the acquisition training were analyzed per day. Cohen'd (Cld) statistic indicated negligible ($0 \leq |d| < 0.2$), small ($0.2 \leq |d| < 0.5$), intermediate ($0.5 \leq |d| < 0.8$), or large ($0.8 \leq |d| \leq 1$) effect sizes (highlighted in bold). There was a significant and large size effect difference between sexes on training Day 1 (for latency to the platform and path length) and on training Day 2 (for path length) but not for training Days 3 and 4. Analyses of variance (ANOVA) effect for sex: *p < 0.05; CV (coefficient of variance), M (male), F (female).

Latency (s)	Mean (± SD)	ANOVA effect	CId	CV	Test for CV
Day 1	M: 24.252 (± 11.21) F: 37.426 (± 15.47)	F(1.34)= 8.559 p = 0.006 *	0.975	M: 0.462 F: 0.413	p = 0.694
Day 2	M: 20.315 (± 11.58) F: 28.176 (± 12.20)	F(1,34)= 3.930 p = 0.056	0.661	M: 0.570 F: 0.433	p = 0.359
Day 3	M: 18,285 (± 11.77) F: 21,663 (± 16.50)	F(1, 34)= 0.126 p = 0.725	0.236	M: 0.644 F: 0.762	p = 0.624
Day 4	M: 13,544 (± 9.64) F: 19,863 (± 16.44)	F(1,34)= 0.560 p = 0.220	0.416	M: 0.711 F: 0.828	p = 0.674
Inversion	M: 23,865 (± 11.46) F: 30,748 (± 16.07)	F(1, 34)= 2.189 p = 0.148	0.493	M: 0.480 F: 0.523	p = 0.775
Path length (cm)	Means (± SD)	ANOVA effect	CId	CV	Test for CV
Day 1	M: 448,727 (± 211.88) F: 648,057 (± 261.51)	F(1,34)= 6314 p = 0.017 *	0.838	M: 0.472 F: 0.404	p = 0.583
Day 2	M: 354,968 (± 180.01) F: 531,753 (± 224.09)	F(1,34)= 6809 p = 0.013 *	0.870	M: 0.507 F: 0.421	p = 0.525
Day 3	M: 314,545 (± 185.33) F: 335,180 (± 179.50)	F(1,34)= 0.115 p = 0.736	0.113	M: 0.589 F: 0.536	p = 0.758
Day 4	M: 229,593 (± 161.93) F: 311,955 (± 144.62)	F(1,34)= 2.590 p = 0.117	0.536	M: 0.705 F: 0.464	p = 0.189
Inversion	M: 439,812 (± 218.23) F: 495,656 (± 214.01)	F(1,34)= 0.601 p = 0.444	0.258	M: 0.496 F: 0.432	p = 0.632

The Fisher's exact test for the platform searching strategies analysis revealed that males used more spatial strategies than females on days 1, 2 and 4 (Fisher's exact test $p < 0.001$, $p < 0.001$, $p = 0.025$, respectively) (Fig. 2**E**).

time spent in each pool quadrant showed a preference for the target quadrant in mice of both sexes, compared to the opposite quadrant [effect for 'sex': $F(1, 34) = 2.214$, $p = 0.146$; 'quadrant': $F(1, 34) = 18.051$, $p < 0.001$; 'sex x quadrant': $F(1, 34) = 0.691$, $p = 0.411$] (Fig. 2**G**).

3.3.4. Platform inversion training

Overall, no significant differences per sex were found in the 'inversion' training acquisition sessions -with the platform displaced to the opposite quadrant- in none of the measures (Fig. 2**C-D**): latency to the platform [effect for 'sex': $F(1, 34) = 2.189$, $p = 0.148$, 'session': $F(5, 170) = 6.041$, $p < 0.001$; 'sex x session': $F(5, 170) = 0.398$, $p = 0.850$], path length [effect for 'sex': $F(1, 34) = 0.601$, $p = 0.444$, 'session': $F(5, 170) = 7.043$, $p < 0.001$; 'sex x session': $F(5, 170) = 0.244$, $p = 0.942$] or velocity -data not shown- [effect for 'sex': $F(1, 34) = 0.339$, $p = 0.564$, 'session': $F(5, 170) = 4.887$, $p < 0.001$; 'sex x session': $F(5, 170) = 0.797$, $p = 0.553$].

The probe trial performed 30 min after the last inversion training session assessed the short-term acquisition of the new platform location. Male and female mice showed a similar latency to reach the platform [$t(34) = -1.085$, $p = 0.285$] and in the number of platform crossings [$t(34) = 0.000$, $p = 1.000$] (Fig. 2**F**). Interestingly, no preference for the target quadrant over the opposite one was found [repeated measures ANOVA' sex x pool quadrant': effect for 'sex': $F(1, 34) = 2.843$, $p = 0.101$; 'quadrant': $F(1, 34) = 3.505$, $p = 0.070$; 'sex x quadrant': $F(1, 34) = 0.673$, $p = 0.418$] in either sex. This suggests that one training day was insufficient for mice to acquire a strong preference for the new target quadrant but, however, could extinguish their preference for the previous one (Fig. 2**G**).

3.3.5. Spatial working memory

Regarding latency in the working memory sessions, both sexes performed equally in the sample sessions where the platform location was unknown [repeated measures ANOVA' sex x day': effect for 'sex': $F(1, 34) = 1.388$, $p = 0.247$; effect for 'day': $F(1, 34) = 3.091$, $p = 0.088$; 'sex x day': $F(1, 34) = 0.444$, $p = 0.510$]. In the test sessions, there was a tendency for males to outperform females, but it did not result statistically significant [effect for 'sex': $F(1, 34) = 3.271$, $p = 0.079$; 'day': $F(1, 34) = 5.840$, $p = 0.021$; 'sex x day': $F(1, 34) = 0.246$, $p = 0.623$] (Fig. 2**H**). Considering the locomotion, there were no sex differences in the simple sessions [repeated measures ANOVA' sex x day': effect for 'sex': $F(1, 34) = 0.0175$, $p = 0.895$; effect for 'day': $F(1, 34) = 7.050$, $p = 0.012$; 'sex x day': $F(1, 34) = 0.000$, $p = 1.000$] nor in the test sessions [effect for 'sex': $F(1, 34) = 2.498$, $p = 0.123$; effect for 'day': $F(1, 34) = 7.050$, $p = 0.012$; effect for 'sex x day': $F(1, 34) = 0.000$, $p = 1.000$] (Fig. 2**I**).

3.4. Hippocampal levels of BDNF did not differ by sex

We evaluated BNDF levels in the hippocampus of female and male mice in two different experiments, under basal conditions (undisturbed, without any exposure to behavioral tasks) and after behavior evaluation. Results yield that behavioral testing increased BDNF expression in the hippocampus of those mice independently of sex [effect for 'procedure': $F(1, 42) = 4.364$, $p < 0.0428$ effect for 'sex': $F(1, 42) = 1.657$, $p = 0.205$]. No interaction between factors was found ['sex x procedure': $F(1, 42) = 0.047$, $p = 0.830$] (Fig. 2**J**).

4. Discussion

This work aimed to study differences between male and female adult C57BL/6 JRj mice in a behavioral test battery for exploratory, emotional and cognitive behavior. Mostly, negative results are reported.

In standard measures of locomotor activity and emotional behavior tested in the EPM, the OFT and the FST, no differences between sexes were found in this study. While depression and anxiety disorders in humans are more common in females than in males, sex differences in rodents tested in the EPM, OFT and FST have been overall ambiguous [35,44]. In previous studies comparing male and female C57BL/6 J mice in 'control' or 'basal' conditions, a certain tendency has been reported for female C57BL/6 J mice to result in more active and less anxious in the OFT or the EPM, with no differences in the FST [29,45–47]. Nevertheless, this outcome seems to strongly depend on numerous variables such as the housing conditions [45,48], the age of weaning [49] or the number of testing trials [47]; which may explain the divergences that exist among studies. For example, both sexes may perform equally in the OF and EPM tasks under standard housing and testing conditions; but sex differences could be exacerbated when C57BL/6 J mice are either housed in an enriched environment [45]; socially isolated during adolescence -but not when isolated during adulthood- [48] or tested during repeated sessions [47]. In addition, the origin of the C57BL/6 J mice (i.e. the vendor) is another factor largely

affecting the behavioral phenotype [32]. To the best of our knowledge, this is the first research on sex differences in the EPM and FST in C57BL/6JRj mice specifically. Previous testing of C57BL/6JRj in the OF test agreed with our data showing no differences by sex in total locomotion and time in the center [50]; tough females reared less than males. Accordingly, we report a strong tendency for female C57BL/6JRj mice to rear less, which could have probably reached statistical signification if our sample was as large as the one used by Sturman et al. [50], which obtained 50 mice per sex after pooling data from several studies.

Regarding memory-related measures, we tested mice in the NOR and NPR paradigms with a 24 h retention interval. These tasks respectively assess '*what*' and '*where*' components of declarative memory and both involve the hippocampus and the medial temporal lobe [51]. However, they have distinct neurobiological basis as evidenced by different ontogenetic development [52]. In humans, sex differences in visual memory are strongly dependent on the material to be remembered. For example, in humans, females show an advantage for images of stimuli that could be named (i.e. entailing a verbal component), faces and locations, while males are more proficient in abstract images [2]. In rodents, variations induced by sex in the NOR and NPR tasks have been unclear [51–53]. Specifically, in male and female C57BL/6 J mice tested in 'basal' conditions, NOR studies have either reported no sex differences (at 5 min ITI) [54]; a female advantage (24 h ITI) [55,56] or a male advantage (3 min ITI) [57]; and no differences have been reported in NPR [54,57]. In the present study, C57BL/6 JRj mice of both sexes equally preferred novel objects and locations over the familiar ones. Interestingly, independently of the memory-related measures, here we report a reduced total exploration of objects in the female C57BL/6 JRj mice which was not explained by a reduced overall exploratory activity, since locomotion through the task was the same in both sexes. This data may suggest that female mice were either less intrinsically motivated to explore objects or that they needed less time to encode their features. A reduced exploration directed to objects in females has been reported in previous C57BL/6 J mice studies [48] but not in the majority [55–57]; and both sexes are intrinsically attracted to certain object traits such as complexity [58]. More research would be needed to confirm whether a reduced object exploration in females could be a trait of the C57BL/6 JRj mouse line specifically; since previous data was obtained in mice acquired from different vendors.

At the end of the behavioral testing battery, male and female C57BL/6 JRj mice were evaluated in the water maze for spatial memory. This task evaluates goal-directed navigation (i.e. "wayfinding": locating oneself and/or a point of reference within the space); a spatial component of declarative memory in humans, in which male participants usually outperform females with small or medium effect sizes [22]. As explained in the introductory section, studies in the water maze have revealed a male advantage in rats, but more research is required to establish conclusions in mice since the existing data are insufficient and controversial [23]. Previous research using C57BL/6 J mice, have failed to reveal differences by sex in the water maze [29,30]. In the present work, it is important to note that pre-training with a visible platform indicated that both sexes had equal abilities (i.e. eyesight, motoric ability, understanding of task rules,…) and motivation (i.e. willing to escape water) to perform the water maze task. In the subsequent spatial reference memory training, females were slower than males in learning the platform location; but both sexes achieved a similar asymptotic performance (i.e. on third and fourth days of training) and did not differ in long-term memory consolidation nor in the spatial working memory task. This suggests that sex differences were limited to a male advantage in spatial reference memory acquisition. Nevertheless, when the platform changed to the opposite quadrant, both sexes acquired this new location equally. This is probably explained because, at that point in testing, animals had previously learned to navigate in the spatial context, which could facilitate the subsequent task for females. Accordingly, previous data in rats reveal that the male advantage is more prominent in protocols that did not use pretraining trials [23], confirming that the difference between sexes could be prevented by variating the testing conditions.

Spatial navigation in the water maze has been traditionally linked to hippocampal function, and in particular to BDNF expression. Depleting hippocampal BDNF in mice impairs spatial memory acquisition in the water maze as well as object recognition memory [59]; and hippocampal BDNF levels are usually directly associated to an improved water maze performance [36,60,61]. However, sex differences in hippocampal BDNF are rarely investigated. Surprisingly, lower hippocampal BDNF levels have been described in male rats compared to females [62] -both basal conditions and after stimulation by environmental enrichment [63]- despite superior spatial performance in males. In this study, we measured hippocampal BDNF in male and female C57BL/6 JRj mice both in basal conditions and five days after completing the behavioral training battery. Behavioral training augmented BDNF in both sexes, and thus it could be understood as an 'enriched' or 'hippocampal-stimulating' treatment compared to undisturbed standard housing; however, no sex differences were found in any experimental condition. A caveat of this data is that it is not possible to know the relationship of each behavioral task to hippocampal BDNF levels, which would require further investigation.

In conclusion, while the literature on basal sex differences in mice is still ambiguous, we reveal C57BL/6 JRj mice as a mouse model potentially suitable to research sex differences in spatial navigation in the water maze. This outcome is valuable considering the scarcity of research in mice that have studied sex differences in spatial navigation tasks revealing a male advantage, as found in human research or in rats. In this regard, it should be noted that, while they initially learned with a delay, female C57BL/6 JRj mice could complete the spatial navigation task as proficiently as males. Thus, female C57BL/6 JRj mice would also be a suitable model for studying the effect of either enhancing or deleterious treatments on spatial navigation. Both sexes did not display any notable differences in other forms of memory, nor in exploratory and anxiety-like behaviors tested in standard paradigms. According to previous statements, it is important to mention that females were not more variable than males in any of the parameters analyzed [16,17,20,21].

Author contributions

Estela Castilla-Ortega and Celia Rodríguez-Pérez conceived and designed the study. Sonia Melgar-Locatelli and M. Carmen Mañas-Padilla conducted the behavioral experiments. Sonia Melgar-Locatelli, Adriana Castro-Zavala, Patricia Rivera and Ana L. Gavito collected the hippocampal samples and performed the western blot. Sonia Melgar-Locatelli, Adriana Castro-Zavala and Estela Castilla-Ortega analyzed the data and wrote the manuscript. All authors critically reviewed the manuscript and approved the final version.

CRediT authorship contribution statement

Sonia Melgar-Locatelli: conceptualization, methodology, formal analysis, investigation, Writing- Original draft preparation, visualization. **M. Carmen Mañas-Padilla:** conceptualization, methodology, investigation. **Ana L. Gavito:** investigation, resources. **Patricia Rivera:** funding acquisition, investigation, supervision. **Celia Rodríguez-Pérez:** funding acquisition, investigation, supervision. **Estela Castilla-Ortega:** funding acquisition, conceptualization, methodology, formal analysis, investigation, writing-original draft preparation, visualization, project administration, supervision. **Adriana Castro-Zavala:** conceptualization, investigation, methodology, formal analysis, writing-original draft preparation, visualization, supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be available from the corresponding authors after reasonable request.

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