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Sex-specific neurobehavioral and biochemical effects of developmental exposure to Malathion in offspring mice

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Abstract

Malathion is an organophosphate pesticide (OP) commonly used in agriculture, industry, and veterinary medicine. Sex is a crucial factor in responding to neurotoxicants, yet the sex-specific effects of OP exposure, particularly neurological impairments following chronic low-level exposure remains limited. Our study aims to evaluate the neurobehavioral and biochemical effects of developmental exposure to Malathion across sexes. Pregnant mice were exposed to a low oral dose of Malathion from gestation up to the weaning of the pups, which were individually gavaged with a similar dose regimen until postnatal day 70. Our results show that Malathion decreased body weight and food intake, reduced locomotor activity and recognition memory. Motor coordination and special memory were only altered in females, whereas we found a male-specific effect of Malathion on social behavior and marble burying. These alterations were accompanied by increased malondialdehyde (MDA), decreased brain acetylcholinesterase activity (AChE), and disrupted brain redox homeostasis. Our findings about the effects of Malathion exposure across sexes may, in part, contribute to understanding the dimorphic susceptibilities observed in neurological disorders.

Keywords Malathion · Neurobehavioral · Chronic exposure · Oxidative stress

Introduction

Biological sex is a crucial factor in responding to neurotoxicants such as organophosphate pesticides (OPs) (Comfort and Re 2017). To this day, there is a considerable amount of evidence elucidating the differences between males and females regarding vulnerability to diseases, xenobiotics, and adverse drug effects (Institute of Medicine (US) Committee on Understanding the Biology of Sex and Gender Differences 2001). Those differences are nearly found in every brain area and are fundamental for sexually dimorphic behaviors and brain damage (Ruszkiewicz et al. 2019). OPs neurotoxicity might range from the sexual differentiation of the brain itself to the prevalence of certain neurological disorders that exhibit sex-dependent outcomes (Comfort and Re 2017; Rauh et al. 2012; Mostafalou and Abdollahi 2017).

For instance, neurodevelopmental impairments such as autism impact males more than females (Ferri et al. 2018), and female-biased conditions include emotional disorders such as depression (Gobinath et al. 2015; Hodes et al. 2017).

Malathion (MAL) is a broad-spectrum OP insecticide extensively used around the globe in agriculture, industry, and veterinary medicine, leading to excessive exposure from multiple sources (Badr 2020). Like other OP compounds, MAL-induced neurotoxicity is due to AChE inhibition, but alone it cannot explain the range of neurological symptoms and disorders following MAL exposure (Kwong 2002; Venkatesan et al. 2017). Non-cholinergic mechanisms such as oxidative stress also play a role in its toxic effects and appear to be correlated with MAL neurotoxicity (Badr 2020). OPs exposure can begin as early as gestation and traverse the placenta and amniotic fluid to the fetus (Bradman and Whyatt 2005), as well as being present in maternal milk (Sanghi et al. 2003). The fetal and child brains are particularly vulnerable at critical periods of growth and development when a major functional organization is being established; OP toxicity can disrupt many processes leading to alteration in the development of the nervous system (Heyer and Meredith

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2017). Moreover, brain sexual differentiation happens during gestation and early adolescence under the instruction of genetic and hormonal factors (Ruszkiewicz et al. 2019). Therefore, this study aims to investigate the sex-specific effects of developmental exposure to MAL, which starts from gestation and lasts until the young adult stage, at a low dose based on a previous study (Ouardi et al. 2019), which mimics the pattern of real-life exposure. All behavioral experiments and biochemical analyses of AChE activity and oxidative stress biomarkers were assessed in male and female mice to answer the recent request posed by the US national institute of health (NIH) to incorporate sex as a biological variable in neuroscience research (McCarthy et al. 2017). Given that most studies in this field have only involved males in their research. The primary reason for scientists to exclude females in animal studies is out of concern for the potential variability introduced by the estrus cycle (Prendergast et al. 2014). In the present study, we only tested females during their non-receptive phase (metestrus and diestrus), thereby minimizing the hormonal factor that may interfere with behavioral phenotypes (Woolley and McEwen 1992; McLean et al. 2012; Moura et al. 2020; Chari et al. 2020). Hence, our study provides a first-time insight into how MAL alters brain biochemicals and behavioral outcomes according to sex while considering the estrus cycle of female mice.

Materials and methods

Chemicals

Commercial-grade Malathion 50 (S-1,2-bis(ethoxycarbonyl) ethyl O,O-dimethyl phosphorodithioate) was purchased. It contains 500 g/liter of Malathion, with the molecular formula $C_{10}H_{19}O_6PS_2$ and a molecular weight of 330.4 g/mol. Acetylthiocholine iodide, 5, 5- dithiobis-(2-nitrobenzoic acid) (DTNB) and all other chemicals used in this study were purchased from Sigma (USA).

Animals and treatment

Adult male and female Swiss albino mice were obtained from the laboratory animal house of the Faculty of Science and Technology, Beni Mellal, Morocco. Mice were accustomed to their room and manipulator for at least two weeks before starting the experiment, the animals were housed in Plexiglas cages under standard conditions, 12-h light/12-h dark cycle, 22 ± 2 °C and 50%—70% humidity, with free access to commercial diet (Alf Sahel, Casablanca, Morocco) and water ad libitum.

Each virgin female mouse and one male breeder were placed in one cage and the vaginal plug was examined daily,

early in the morning. Once the vaginal plug was detected mice were housed individually and randomly divided into two groups of 10 animals each: Group 1 received only the vehicle (5% crone oil, 95% soja oil). Group 2 received MAL dissolved in oil at the dose of 15 mg/kg of body weight per day. The treatment was orally administered by intragastric gavage and lasted throughout gestation and lactation until post-partum day 21, after which weaned pups of each experimental group were submitted to the same regimens of the preliminary protocol. The duration of treatment was extended to the young adult stage. The dose of malathion used in this study, as well as the treatment duration choice, is based on previous work (Ouardi et al. 2019; N'Go et al. 2013a). All experimental procedures performed on animals are in accordance with the scientific procedures of living animals reported by the European Council directive: ACT: 86/609 EEC. The experimental design of our study is summarized in Fig. 1.

Body weight and food intake

The body weight of offspring was recorded once a week from PND 21 until PND 70. At the end of treatment, mice were housed individually per cage and had free access to a normal diet (30 g/cage/day) and water. The experiment lasted 4 weeks, totaling 7 animals/group, the first week serves as the habituation period. At day 0, all animal groups started with an average weight of 19 g (females) and 24 g (males). Every day at 9 AM, the animals' body weight and water intake were recorded, and the rations per cage that were not consumed were weighed and replaced with new pellets (30 g) (Fig. 2B). Food intake was measured as the difference in pellets weight over 24 h (Ferreira-Paes et al. 2021).

Estrous cycle stage detection

Estrus cycle stage determination was done using vaginal cytology which is a non-invasive method proposed by Ashleigh C. Mclean (McLean et al. 2012). In the morning (between 8:00 AM and 9:00 AM) before behavioral tests, stages of the estrus cycle were detected by examining the vaginal smear under light microscopy. The vaginal cells were flushed with small amounts of distilled water through a sterile pipette and then the droplets of cell suspension were placed in a glass slide to dry on a Hot Plate. For microscopic examination, 0.1% crystal violet was prepared for staining, by adding 0.1 g of crystal violet powder in 100 ml of distilled water. The vaginal secretions are normally composed of three types of cells namely leucocytes, cornified epithelial cells and nucleated epithelial cells. The proportion of cell types present in the vaginal smear will determine which estrous stage is taking place.
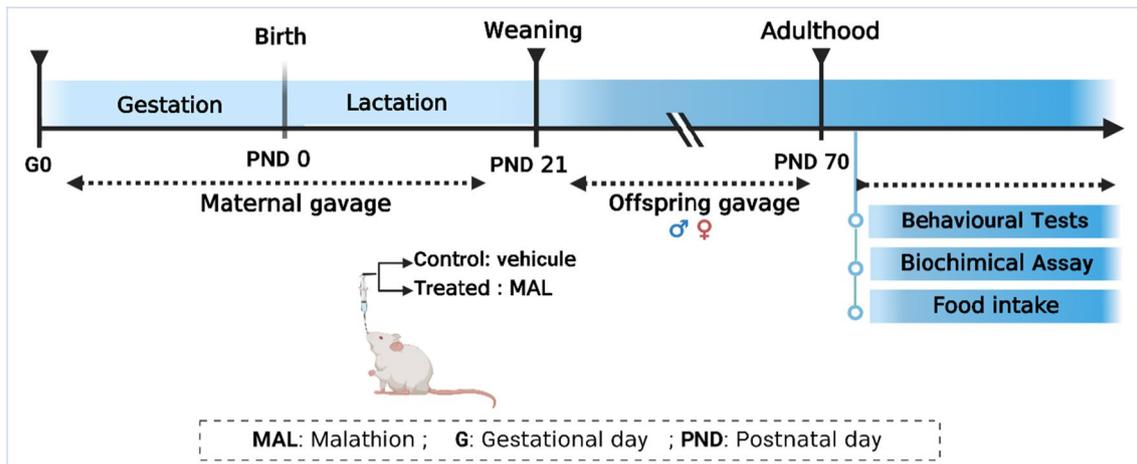
Fig. 1 Experimental design and animal treatment

Behavioral tests

Twenty-four hours after the final MAL exposure, mice (aged 10 weeks) were subjected to behavioral testing from the least to most stressful ones in the following order: Open Field, Novel Object Recognition, Y-maze, Social Interaction, Marble Burying, Rotarod, Tail Suspension. All mice were acclimated in the testing room for 30 min before each behavioral test, and testing occurred between 9:00 AM and 1:00 PM (first half of the light cycle). After each behavioral test, mice were left to rest for at least one day. Female mice were tested during the metestrus and diestrus phases of the estrous cycle, which are characterized by low levels of estradiol and progesterone, to prevent any hormone-induced effects that may modulate behavioral outcomes (Woolley and McEwen 1992; McLean et al. 2012; Moura et al. 2020; Chari et al. 2020). Male and female mice behavioral tests were conducted on separate days to account for sensitivity to pheromones. The apparatus was cleaned between tests with a solution of ethanol 10% to prevent any bias due to olfactory cues.

Open files test

The open-field test (OF) was used to assess general locomotor activity levels. The apparatus ($50 \times 50 \times 50$ cm) is made of black Plexiglas and a black bottom subdivided into 25 squares. Each mouse was placed in the center of the OF and recorded for 5 min using a video camera located above the OF and monitored in another room via a closed-circuit TV camera. The levels of general activity were measured with two parameters, the total distance traveled and average speed. Data were analyzed using ANY-Maze software (Stoelting Co., Wood Dale, IL, USA).

Novel object recognition test

The novel object recognition test is a commonly used test to evaluate general memory function in rodents (Yin et al. 2011). The apparatus used in this test is an open-field-testing arena ($50 \times 50 \times 50$ cm) made of black Plexiglas. The test procedure consists of three main sessions conducted over two days. The habituation session (day 1), where each animal was allowed to freely explore the empty arena for 5 min. During the training session (day 2), the animals are exposed to the familiar arena with two identical objects placed at an equal distance of 10 cm away from the walls for 5 min. Two hours later, the test session began by allowing mice to explore the OF for 5 min in the presence of a familiar object and a novel object to test short-term recognition memory. An active investigation was set as when a mouse engages an object with its nose pointed at the object no more than 2 cm away. The time spent exploring each object was recorded and the discrimination index was calculated [((time spent beside the novel object – time spent beside the familiar object) / total duration beside both objects) $\times$ 100] (Ennaceur and Delacour 1988).

Y-maze test

Spatial working memory was assessed using the spontaneous alternation Y-maze test as described previously by Malqui (Malqui et al. 2018). The apparatus was made of Plexiglas with three identical arms ($40 \times 9 \times 16$ cm) placed 120° from each other. Ten-week-old mice were placed in one of the arms and were allowed five minutes of free exploration in the three arms of the Y-maze. The series of arm entries (e.g., ABC, BCA) and total arm entries were recorded using an overhead camera and analyzed by ANY-Maze software.
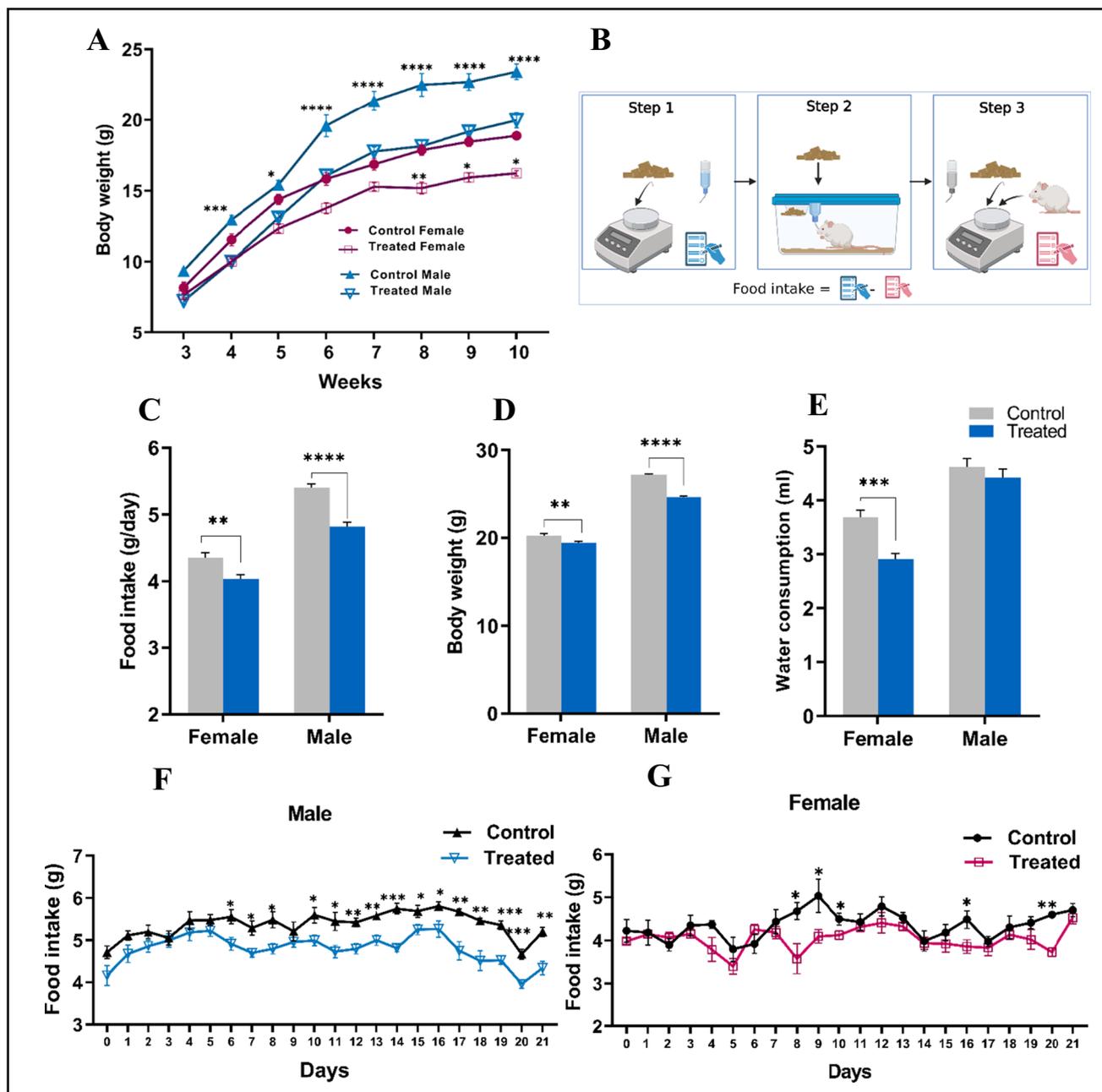
Fig. 2 Evaluation of food intake, water intake and body weight in mice offspring exposed to MAL from in-utero until young adult age. **A** Body weight progression from week 3 of life in male and female offspring. **B** Food intake protocol. **C**, **D**, **E** Body weight and 24-h food and water intake were measured continuously for 21 days in all male and female mice, $n = 7$ for each control and treated group. **F**, **G** 24-h food intake in males and females were measured daily in all groups and the data are presented in panels **F** and **G**, respectively. All results are presented as mean ± SEM. **A** Comparing body weight as analyzed by three-way repeated measures ANOVA. **C**, **D**, **E** Comparing body weight/food intake/water intake as analyzed by two-way ANOVA. **F**, **G** Comparing food intake/body weight in treated and controls as analyzed by student's paired t test. $*p \leq 0.05$; $**p \leq 0.005$; $***p \leq 0.001$; $****p \leq 0.0001$

The percentage of spontaneous alternations was calculated as an index of working memory performance (index of alternation).

Social interaction test

Crawley's sociability and preference for social novelty test apparatus is comprised of a rectangular, three-chamber box. Each chamber is 20×45 cm and the dividing walls are made

of clear removable Plexiglas, with an open middle section, which allows free access to each chamber. Both left and right chambers contain an empty wire cup in the center. The test is divided into three different phases (habituation, sociability and social novelty). In the habituation phase, the mouse was placed in the center chamber with no access to both compartments and allowed 5 min of exploration. In the sociability phase, a novel mouse (stranger 1) was enclosed in one of the cups and the test mouse was allowed to explore all three chambers for 10 min. The novel mouse was of the same sex, age, and strain and was not a sibling of the test mouse. In the social novelty phase, a new novel mouse (stranger 2) was placed under the cup of the other side and tested again for 10 min. The time spent in each chamber and the time spent exploring enclosed novel mice or empty cups (novel objects) were recorded in the 10 min sessions. Active contact was defined as when a test mouse stretches toward the cup with a distance of 3–5 cm around the cup or as climbing on the cup (Crawley 2004). Preference for novel mouse 1 was calculated as [(time spent exploring novel mouse)/ (total time spent exploring novel mouse and novel object)] × 100%. And the preference for novel mouse 2 was calculated as [(time spent exploring novel mouse 2)/ (total time spent exploring novel mouse 1 and novel mouse 2)] × 100% (Lo et al. 2016).

Marble burying test

The Marble Burying test was carried out as previously described by Angoa-Pérez (Angoa-Pérez et al. 2013) with minimal modifications. Briefly, mice were individually placed in a mouse cage (47 cm length × 23 cm width × 15 cm height) with 20 glass marbles evenly spaced on a 5 cm deep layer of wood ship bedding lightly pressed to give a flat surface. Each mouse was placed in the corner of the cage containing marbles and allowed 30 min to explore freely; the number of marbles buried by each animal was counted. A Marble was considered buried if 2/3 or more of its volume was submerged.

Rotarod test

The Rotarod test was used to assess motor coordination in mice as described by Dunham and Miya (Dunham and Miya 1957) with some minor modifications. The device used is mouse Rota-Rod NG (Ugo basile, Gemonio, Italy). During testing, when a mouse falls from its cylinder section on a box below, a magnetic switch is activated by pooling the plate box, thus recording the animal's endurance time in seconds. The test consists of three trials separated by inter-trial intervals of 15 min, with the speed accelerated from 4 to 40 rpm for 300 s. The latency at which each mouse falls from the rod is recorded.

Tail suspension test

The animals were suspended individually, with adhesive tape placed approximately 1 cm from the tip of the tail, on a shelf with a height of 15 cm from the bottom of the testing setup. The duration of immobility was recorded for 6 min. Immobility is defined as the cessation of any bodily movements (Yin et al. 2011).

Biochemical assays

Sample collection

The brains of the control and MAL-treated mice were collected ($N = 7$/experimental group) by decapitation upon completion of the treatment period. Each of the following brain structures, olfactory bulbs, hippocampus (right and left sides), hypothalamus and cerebellum were rapidly dissected on a plate at 4 °C, weighed, and frozen at −20 °C until use.

Tissue preparation

Brain structures were homogenized in buffer solution TBS (50 mM Tris, 150 mM NaCl, pH 7.4); and then centrifuged at 10,000 × g for 15 min at 4 °C with centrifuge 5804 R (Eppendorf, Freshwater Blvd Enfield, USA). Supernatants were carefully collected and used for the determination of AChE Activity, antioxidant enzymatic markers (catalase, superoxide dismutase, glutathione peroxidase) and lipid peroxidation marker (malondialdehyde). Protein concentration was assayed by the method of Lowry (Lowry et al. 1951) with bovine serum albumin as the standard.

Acetylcholinesterase activity (AChE)

The AChE specific activity was measured according to the method of Ellman (Ellman et al. 1961) using acetylthiocholine iodide (Sigma-Aldrich, USA) as a substrate. The reaction mixture contained phosphate buffer (0.1 M, pH 8.0), acetylthiocholine iodide (0.075 M), and 5,5- dithio-bis-2-nitrobenzoic acid (DTNB; 0.01 M) (Sigma- Aldrich, USA). After the addition of brain structure tissue homogenate (olfactory bulb, cerebral cortex, or brainstem) (30 min at room temperature), the hydrolysis rate of acetylcholine iodide was measured by a spectrophotometer (Selecta, Barcelona, Spain) at 412 nm. The enzyme activity was expressed as µmol Ach hydrolyzed/min/mg of protein.

Malondialdehyde (MDA)

The MDA activity in the brain was measured by the method of Buege and Aust (Buege and Aust 1978). A total of 125 µl of supernatant was homogenized by sonication with 50 µl of

PBS and 125 µl of 20% TCA + 1% BHT (TCA-BHT), to precipitate proteins, and centrifuged ($1000 \times g$, 10 min, 4 °C). Afterwards, 200 µl of supernatant was mixed with 40 µl of HCl (0.6 M) and 160 µl of TBA dissolved in Tris (120 mM). The mixture was heated at 80 °C for 10 min, and the absorbance was measured at 530 nm. The amount of thiobarbituric acid reactive substances (TBARS) was calculated using a molar extinction coefficient of 1.56×10^5 M/Cm.

Catalase (CAT)

The CAT activity was measured at 240 nm using a UV/visible spectrophotometer by the variation of the optical density consecutive to the disproportionation of hydrogen peroxide (H_2O_2). For the enzyme reaction, 20 µl of supernatant was added to 780 µl of phosphate buffer saline (PBS) (0.1 M, pH 7.4) and 200 µl of H_2O_2 (0.5 M) (Aebi 1974).

Superoxide dismutase (SOD)

The SOD activity was measured as described by Asada (Asada et al. 1974); 0.05 ml of the supernatant was added to 0.1 ml of a mixture containing methionine (13 mM) and Na_2EDTA (0.1 mM), 0.8922 ml of phosphate buffer (50 mM, pH = 7.8), 0.95 ml of phosphate buffer, 0.088 ml of NBT (2.64 mM), and 0.0226 ml of riboflavin (0.26 mM). The reduction of NBT was estimated after 20 min at a wavelength of 580 nm against white.

Glutathione peroxidase (GPx)

For each assay, a mixture containing 200 µl of supernatant, 200 µl of phosphate buffer (100 mM), 200 µl of 4 mM GSH, and 400 µl of H_2O_2 (5 mM) was incubated for 1 min at 37 °C. After adding 500 µl of 5% TCA, the mixture was then centrifuged for 5 min at $1500 \times g$. Two hundred microliters of the supernatant was recovered, 500 µl of phosphate buffer and 500 µl of DTNB were added, and the absorbance was measured at 412 nm each min for 5 min according to Flohé and Günzler (Flohé and Günzler 1984) modified method.

Data analysis and statistics

Statistical analysis was performed using parametric tests since all data was normally distributed. The two-way ANOVA was used for all data analysis considering MAL treatment and sex as fixed factors, except for body weight and the time spent in the three-chamber test that was analyzed by three-way ANOVA. Tukey's Post hoc test was used for all data multiple comparisons used in this study. In addition, data from the daily three-week food intake curves were analyzed by student's paired *t-test* using SigmaPlot 11 software (Systat Software Inc, California, US). All data were

expressed as the mean ± SEM. Differences were considered statistically significant at $p \leq 0.05$. GraphPad Prism 9.0 was used for data analysis and figure generation.

Results

Body weight and food intake

Exposure to 15 mg/kg of MAL did not significantly affect maternal weight compared to controls (data not shown). Offspring's body weight progression during the seven weeks of treatment was assessed by three-way ANOVA (treatment × sex × Time) with repeated measures. We found a significant effect of treatment ($F_{(1, 323)} = 304.2$, $P < 0.0001$), sex ($F_{(1, 323)} = 289.1$, $P < 0.0001$), and time ($F_{(7, 323)} = 394.3$, $P < 0.0001$) with an interaction between sex × Treatment ($F_{(1, 323)} = 18.60$, $P < 0.0001$), but without interaction between all factors ($F_{(7, 323)} = 0.45$, $P = 0.86$) in all groups. To further study the weekly development of body weight, a multiple comparison test (Tukey's post hoc test) was used to assess the weekly differences between groups. MAL-treated males presented a permanent effect of the treatment from week 4 onwards, whereas in MAL-treated females this effect was observed from week 8 onwards (Fig. 2A). These results showed apparent sex- and time-dependent effects of MAL on body weight.

Because the effect on body weight (shown in Fig. 2A) could reflect underlying differences in food intake, we assessed food consumption in mice offspring (aged 10 weeks) exposed to MAL from in-utero until young adult age (Fig. 2B). As shown in Fig. 2F, G, MAL-treated male and female mice had distinct food consumption patterns, specifically female mice that showed a different and fluctuating profile of food intake over the period evaluated. While male-treated mice showed a significant decrease in food intake from day 6 until the end of the experiment ($t = 2.822$, $P = 0.018$), females did not exhibit any significant changes in food intake except for a few days throughout the experiment starting from day 8 ($t = 2.879$, $P = 0.015$). The Two-way ANOVA showed a main effect of treatment ($F_{(1, 80)} = 49.08$, $P < 0.0001$) and sex ($F_{(1, 80)} = 199.8$, $P < 0.0001$) with an interaction ($F_{(1, 80)} = 4.10$, $P = 0.046$) on food consumption in all groups. The food consumption was higher in males than in females during the period evaluated (Males: 5.110 ± 0.0637 g, Females: 4.197 ± 0.052 g; Two-way ANOVA analysis, mean of three weeks) as males had a higher body weight and therefore a higher energy demand. Moreover, the multiple comparison test also showed a significant decrease in food intake in treated male ($P < 0.0001$) and female ($P = 0.0039$) groups compared to controls (Fig. 2C). During this experiment body weight and 24-h water intake were

also recorded daily. We found a significant main effect of treatment on body weight (F (1, 80) = 125.6, $P < 0.0001$), and sex (F (1, 80) = 162.6, $P < 0.0001$) with an interaction treatment × sex (F (1, 80) = 36.13, $P < 0.0001$). In addition, the post hoc test also showed a significant decrease in body weight of male ($P < 0.0001$) and female ($P = 0.0024$) treated groups compared to controls (Fig. 2D). For water intake, we found a significant difference among the factor treatment (F (1, 80) = 12.47, $P = 0.0007$), and sex (F (1, 80) = 79.28, $P < 0.0001$) with the interaction between the two factors (F (1, 80) = 4.450, $P = 0.038$). However, the post hoc comparison test showed a significant decrease in water intake in treated female mice only ($P = 0.0008$) compared to controls (Fig. 2E). These data demonstrate that chronic MAL exposure alters feeding behavior in both sexes, with a stronger MAL response on male mice than females,

characterized by a stronger decrease of food consumption correlated with a reduction of body weight.

Behavioral tests

Locomotor activity and motor coordination

Both treated male and female mice displayed decreased locomotor activity, as shown by the trajectories and heat plots mapping of the occupied areas in the OF test (Fig. 3A). Indeed, the two-way ANOVA analysis showed that the treatment with MAL had an obvious effect on locomotor activity in both sexes as measured by the total traveled distance (F (1, 28) = 24.23, $P < 0.0001$) and average speed (F (1, 30) = 25.06, $P < 0.0001$) in the OF test. However, we found no effect of sex or the interaction between
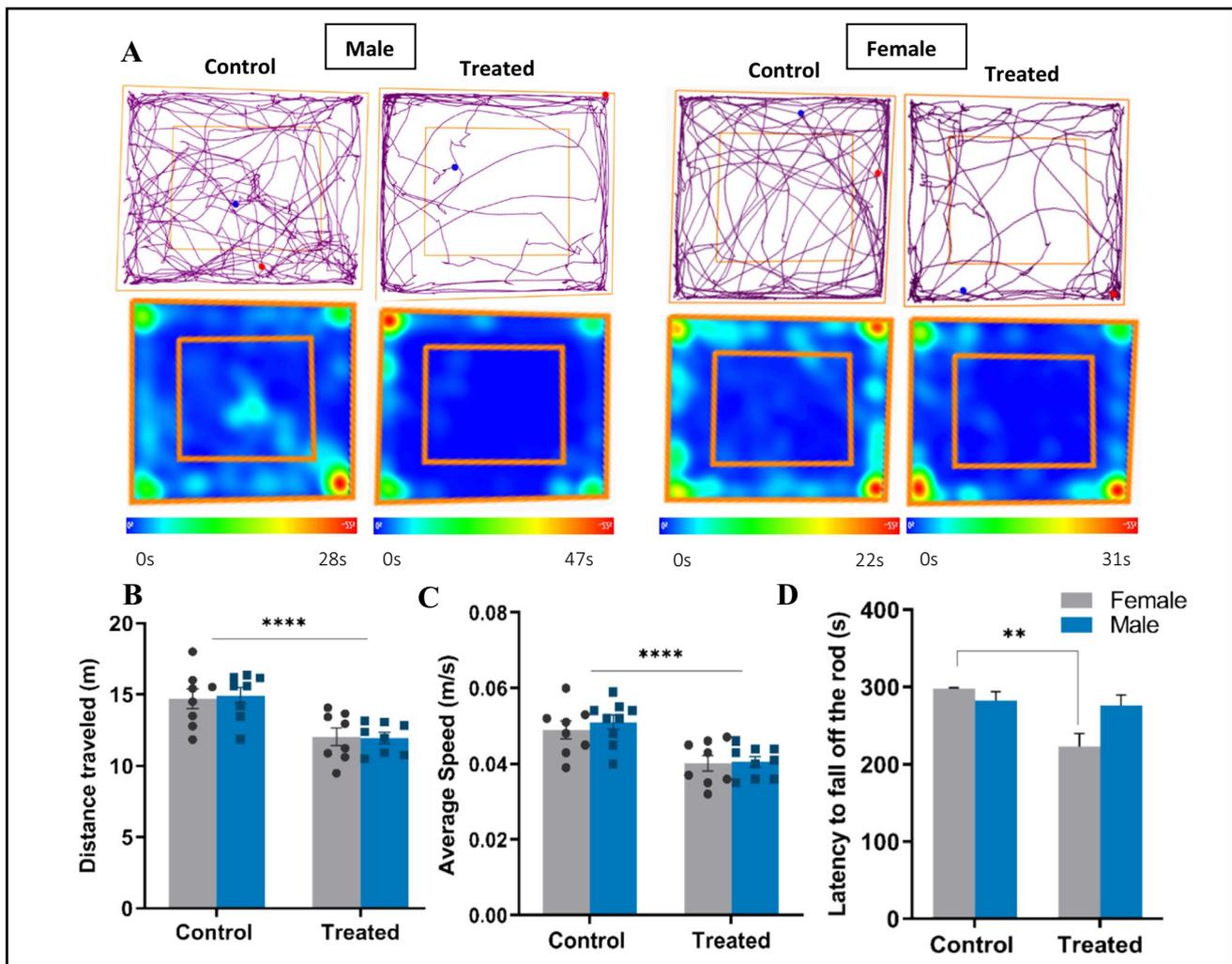

Fig. 3 Effect of MAL chronic exposure on locomotor activity and motor coordination. **A** Trajectories and heat plots mapping the occupied areas in the OF test. **B** Total distance traveled and. **C** Average speed in the OF test. **D** Latency to fall in the rotarod test. **A** Time and color scales are indicated below heat plots. **B**, **C**, **D** Results are expressed as mean ± SEM. Significant effects were revealed by two-way ANOVA and Tukey's test. **$p \le 0.005$; ****$p \le 0.0001$

factors in both parameters used to assess locomotor activity. Moreover, the post hoc analysis showed that the traveled distance and average speed significantly decreased in treated female ($P = 0.014$; $P = 0.018$; respectively) and male ($P = 0.005$; $P = 0.002$; respectively) mice compared to their controls (Fig. 3B, C).

To assess motor coordination, the length of time (second) that the mice remained on the rod was measured in Rotarod test. A two-way ANOVA analysis of this test demonstrated a significant main effect of MAL treatment ($F (1, 24) = 11.15$, $P = 0.0027$) on the latency to first fall, as well as a significant treatment × sex interaction ($F (1, 24) = 7.905$, $P = 0.0097$). However, the sex factor ($F (1, 24) = 2.298$, $P = 0.14$) did not affect the performance in this test. The post hoc multiple comparison analysis showed that MAL treatment significantly decreased the latency to fall off the rod in female mice compared to their controls ($P = 0,0012$) but not in male mice (Fig. 3D).

Learning and memory

The novel object recognition test indicated that chronic exposure to MAL affected recognition memory in adult mice. The two-way ANOVA analysis revealed a significant main effect of treatment on the discrimination index ($F (1, 29) = 41.38$, $P < 0.0001$), but without a sex effect ($F (1, 29) = 3.29$, $P = 0.08$) or an interaction between MAL treatment and sex ($F (1, 29) = 0.69$, $P = 0.41$). Both treated male and female groups exhibited lower discrimination index in comparison to their control groups as showed by the post hoc analysis ($P = 0.004$; $P < 0.0001$; respectively) (Fig. 4A).

Regarding the Y-maze test, chronic exposure to MAL affected short-term spatial working memory in treated mice. Indeed, two-way ANOVA analysis pointed out a

significant difference among the factor treatment ($F (1, 25) = 28.67$, $P < 0.0001$) and the interaction treatment × sex ($F (1, 25) = 4.94$, $P = 0.03$), but not in the sex factor ($F (1, 25) = 0.07$, $P = 0.78$). The post hoc test indicated that the treatment significantly decreased the percentage of alternation in females ($p < 0.0001$) but not in male mice, indicating a short-term memory impairment, especially in the female-treated group (Fig. 4B).

Depression-like behavior

The two-way ANOVA analysis revealed a significant main effect of chronic MAL treatment ($F_{(1, 30)} = 30.11$, $P < 0.0001$) on immobility time evaluated in the tail suspension test, with no effect of sex ($F_{(1, 30)} = 0.4450$, $P = 0.5$) or the interaction between factors ($F_{(1, 30)} = 0.003$, $P = 0.95$). On the other hand, the multiple comparison test showed that MAL treatment increased immobility time more so in females ($P = 0.0009$) than males ($P = 0.0069$) compared to their controls (Fig. 4C).

Marble burying behavior

The Marble burying task has been widely applied to asses repetitive and perseverative behaviors for characterizing autism-related behaviors (Thomas 2009; Silverman et al. 2010). The two-way ANOVA analysis demonstrated a significant effect of all three factors, MAL treatment ($F (1, 24) = 17.68$, $P = 0.0003$), sex ($F (1, 24) = 8.75$, $P = 0.006$) and a significant sex × treatment interaction effect ($F (1, 24) = 6.82$, $P = 0.015$), on the number of buried marbles. In addition, the post hoc analysis showed that male-treated mice buried a significant number of marbles ($P = 0.0004$) than did control males, unlike females displaying no difference
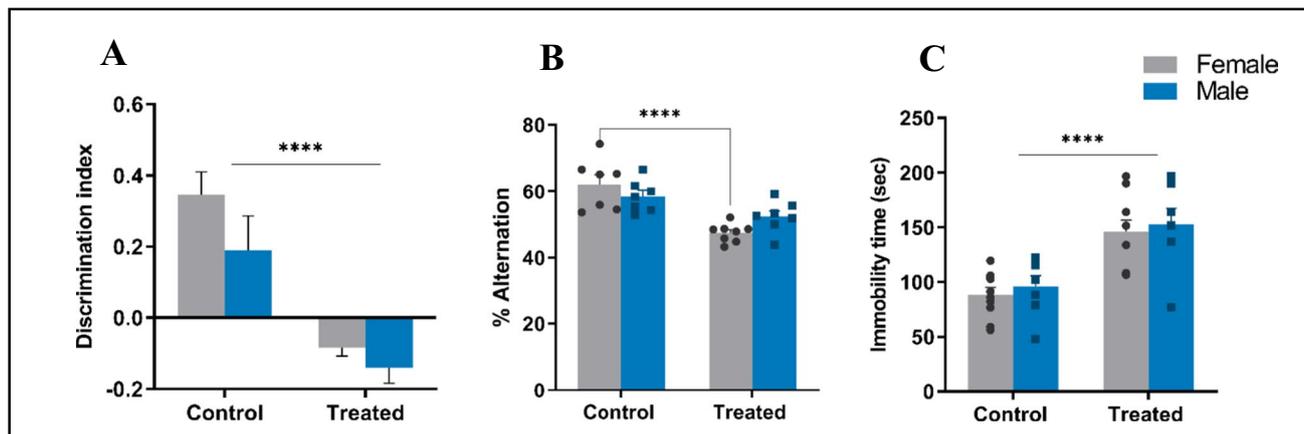

Fig. 4 Effect of MAL on memory function and depressive-like behavior. **A** Recognition memory evaluated by the object recognition test. **B** Spatial working memory evaluated by Y-maze test. **C** Immo-bility time recorded in the tail suspension test. **B**, **C**, **D** Results are expressed as mean ± SEM. Significant effects were revealed by two-way ANOVA and Tukey's test. ****$p \leq 0.0001$

between treated and control groups (Fig. 5E). Suggesting a male-specific effect of MAL treatment on marble burying, which elucidate some traits of autistic-like behaviors.

Social interaction and preference to social novelty

In general, autistic individuals are known to have reduced or unusual social behaviors (Gupta et al. 2020). In our study, we used a three-chamber apparatus to asses sociability and social novelty preference in MAL-treated mice. In the sociability test, the time spent in each side chamber containing a novel mouse or empty wire cup (novel object) was recorded. The three-way ANOVA analysis showed that neither the factor treatment nor sex had an effect ($P > 0.9999$), but pointed a significant difference for the chamber factor ($F (2, 60) = 171.0$, $P < 0.0001$) and chamber × treatment × sex

interaction ($F (2, 60) = 7.94$, $P = 0.0009$) (Fig. 5A). As for the preference for novel mouse, the two-way ANOVA showed a significant main effect of treatment ($F (1, 20) = 5.24$, $P = 0.033$) as well as the interaction treatment × sex ($F (1, 20) = 4.36$, $P = 0.049$), but not of the sex factor ($F (1, 20) = 3.42$, $P = 0.079$). Moreover, male treated mice showed 40% preference for the novel mouse, which is ~20% less than control male mice (~60%) ($P = 0.043$ by Tukey's post hoc test; Fig. 5C). Females, on the other hand, had comparable sociability in both treated and control groups. This data suggests a male-specific effect of MAL treatment on social behavior.

In the social novelty test, the time spent in each chamber containing the novel mouse 1 (familiar mouse), or the novel mouse 2, or the center chamber was recorded. The three-way ANOVA analysis showed no effect of all factors;
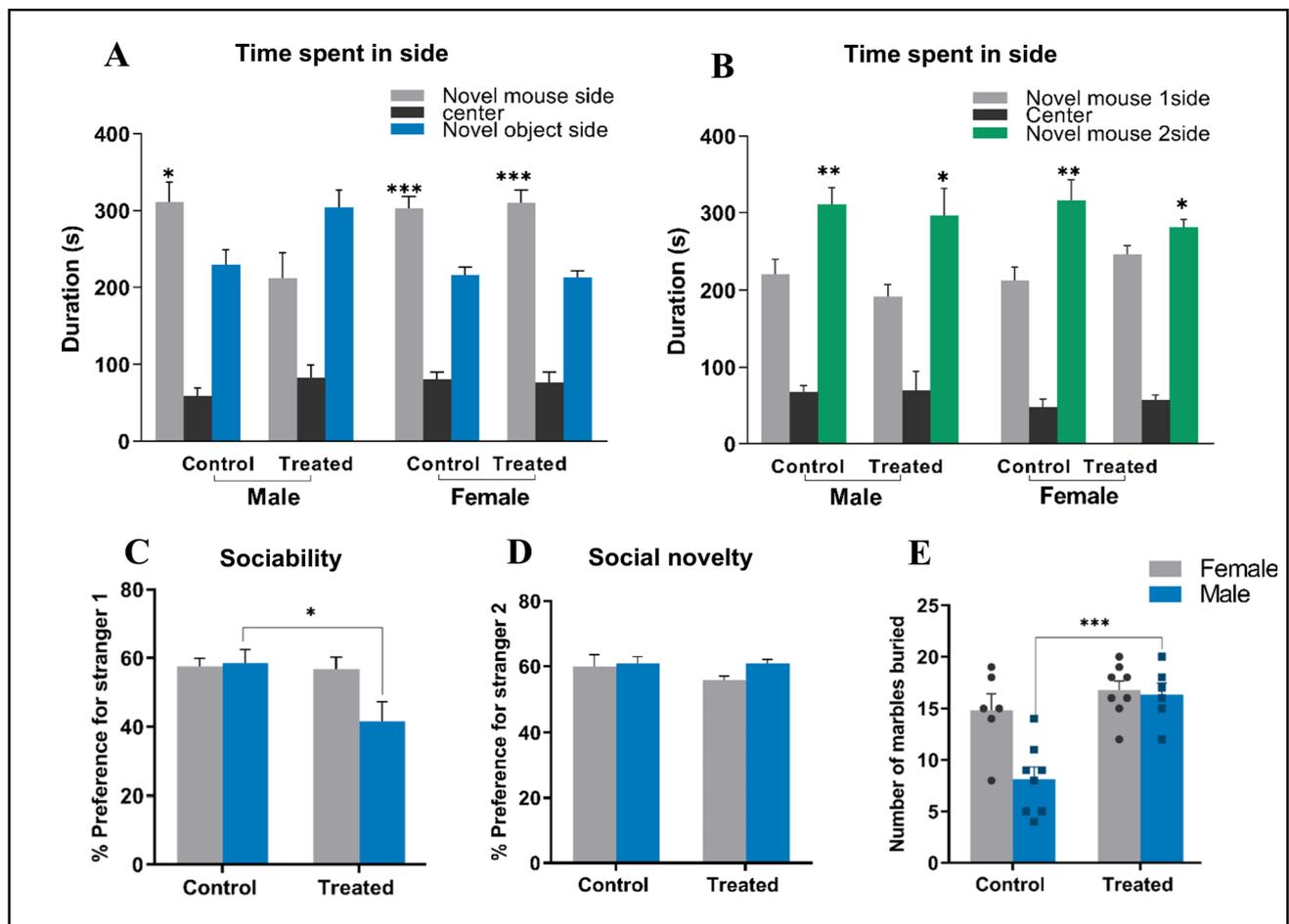

Fig. 5 Effect of MAL on sociability and preference for social novelty in the three-chamber test and marble burying test. **A**, **C** Sociability test. Graphs show **A** time spent in each side chamber containing novel mouse or empty wire cup (novel object), or the center chamber; **C** preference for stranger 1. **B**, **D** Social novelty test. Graphs show **B** time spent in each side chamber containing stranger 1 (now familiar), or stranger 2, or the center chamber; **D** preference for stranger 2. **E** Marble burying test. Graphs show results as mean ± SEM. For treatment effect within each sex for **C, D, E**, by Tukey's post-hoc tests following two-way ANOVA with the factors of treatment and sex; for chamber effect between novel mouse vs. novel object in (**A**), and for stranger 1 vs. stranger 2 in (**B**), by Tukey's post-hoc test following three-way ANOVA with the factors of treatment, sex and chamber side. *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$
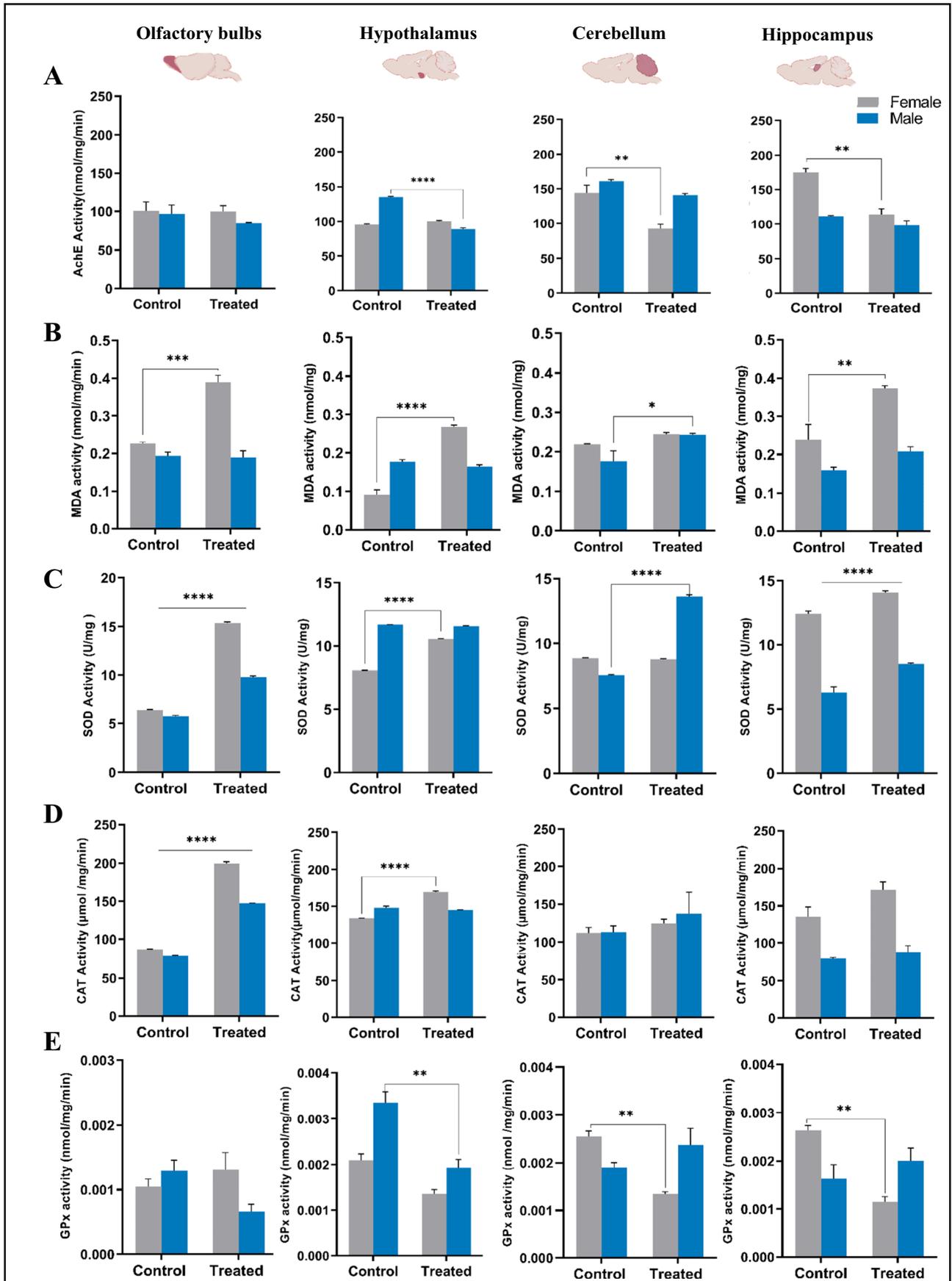
◄**Fig. 6** Effect of MAL treatment on AChE and oxidative stress in olfactory bulbs, hypothalamus, cerebellum and hippocampus. (A) AChE activity. (B) MDA activity. (C) SOD activity. (D) CAT activity. (E) GPx activity. Results are presented as mean ± SEM. Significant effects were revealed by Tukey's test following two-way ANOVA. *$p \leq 0.05$; **$p \leq 0.005$; ***$p \leq 0.001$; ****$p \leq 0.0001$

treatment, sex, chamber × Treatment × sex interaction, except the chamber factor (F (2, 60) = 184.0, $P < 0.0001$), on the time spent in each chamber (Fig. 5B). As for the preference for stranger 2, the two-way ANOVA analysis also showed no significant difference in all three factors (treatment, sex, and treatment × sex interaction) (Fig. 5D). All experimental animals of both sexes showed a clear preference for investigating novel mouse 2 over the familiar novel mouse 1. Thus, these results suggest that MAL-treated mice, regardless of sex have normal social recognition for familiar versus novel mice, as well as a preference for newer social stimuli.

Biochemical assays

Acetylcholinesterase activity

The data analysis showed that chronic MAL exposure induced a global reduction of AChE activity in almost all brain structures (Fig. 6A). In the cerebellum, the two-way ANOVA showed a significant effect of all factors, treatment (F (1, 8) = 29.62, $P = 0.0006$), sex (F (1, 8) = 24.40, $P = 0.0011$), and an interaction sex × treatment (F (1, 8) = 5.75, $P = 0.043$). The post hoc test revealed a significant decrease in AChE activity in females ($P = 0.0024$) but with no significance in male groups showing only a small tendency ($P = 0.21$). The same goes for the hippocampus, where we found an effect of treatment, sex and the interaction sex × treatment (F (1, 8) = 34.92, $P = 0.0004$; F (1, 8) = 40.26, $P = 0.0002$; F (1, 8) = 14.22, $P = 0.0055$, respectively). Also, the post hoc test revealed a significant inhibition of AChE activity in treated females ($P = 0.0006$) and a slight tendency in treated males ($P = 0.47$) compared to their sex-matched vehicle controls. For the hypothalamus, we also obtained a significant effect of all factors (F (1, 8) = 302.7, F (1, 8) = 141.9, F (1, 8) = 444.3, respectively and ($P < 0.0001$) similar in all factors). The multiple comparison test showed a tendency in MAL-exposed females ($P = 0.11$) and a significant decrease in AChE activity in treated males ($P < 0.0001$) compared to controls. As for the olfactory bulbs, no statistical differences were observed between groups.

Lipid peroxidation activity

MAL-exposed mice presented a general increase in MDA activity in all brain structures (Fig. 6B). Within the olfactory

bulbs, the two-way ANOVA revealed a significant effect of both factors, treatment (F (1, 8) = 34.84, $P = 0.0004$) and sex (F (1, 8) = 74.64, $P < 0.0001$) with an interaction sex × treatment (F (1, 8) = 38.23, $P = 0.0003$). The post hoc test showed a significant increase in MDA activity in female-treated mice ($P = 0.0001$) but not in male mice. In the cerebellum, we obtained a significant effect of treatment (F (1, 8) = 12.34, $P = 0.0079$), but with no effect of sex or the interaction between factors. The post hoc test revealed a significant increase in MDA activity only in the male-treated group ($P = 0.0299$) compared to controls. The hippocampus MDA activity analysis demonstrated a main effect of both factors, treatment (F (1, 8) = 18.38, $P = 0.0027$) and sex (F (1, 8) = 33.75, $P = 0.0004$) but without an interaction sex × treatment. The multiple comparison test showed a significant increase in MDA activity in female-treated mice and not in male-treated mice compared to controls. As for the hypothalamus, we obtained a significant effect of treatment (F (1, 8) = 115.6, $P < 0.0001$) and the interaction between sex × treatment (F (1, 8) = 154.4, $P < 0.0001$) but with no effect of sex. The post hoc test showed, once again, a significant increase in MDA activity in females but not in male-treated groups compared to controls.

Antioxidant enzyme activity

The data analysis showed that chronic MAL exposure induced biochemical alteration in all brain structures. Concerning the SOD activity (Fig. 6C), the two-way ANOVA revealed a significant effect of treatment, sex, and the interaction treatment × sex in all brain structures (olfactory bulbs: F (1, 8) = 5028, $P < 0.0001$; F (1, 8) = 1178, $P < 0.0001$; F (1, 8) = 714.1, $P < 0,0001$; hypothalamus: F (1, 8) = 1382, $P < 0.0001$; F (1, 8) = 5185, $P < 0.0001$; F (1, 8) = 1662, $P < 0.0001$; cerebellum: F (1, 8) = 1258, $P < 0.0001$; F (1, 8) = 446.3, $P < 0.0001$; F (1, 8) = 1336, $P < 0.0001$; hippocampus: F (1, 8) = 56.85, $P < 0.0001$; F (1, 8) = 515.1, $P < 0.0001$; with no interaction between factors). Overall, the post hoc analysis revealed a significant increase in SOD activity in the hippocampus (males: $P = 0.0012$, females $P = 0.0090$) and olfactory bulbs ($P < 0.0001$) of both sexes. However, a significant increase in SOD activity was only found in the cerebellum of male-treated mice ($P < 0.0001$) and the hypothalamus of female-treated mice compared to controls ($P < 0.0001$).

Also, there was an increase of CAT activity in the olfactory bulbs and hypothalamus, but no differences were found in the hippocampus and cerebellum (Fig. 6D). As revealed by the two-way ANOVA there was a significant effect of treatment, sex, and interaction (olfactory bulbs: F (1, 8) = 5123, $P < 0.0001$; F (1, 8) = 552.3, $P < 0.0001$; F (1, 8) = 298.7, $P < 0.0001$; hypothalamus: F (1,

8) $= 157.8$, $P < 0.0001$; $F (1, 8) = 14.53$, $P = 0.0051$; $F (1, 8) = 223.4$, $P < 0.0001$). The post hoc test confirmed the increase of CAT activity in both males and females relative to their sex-matched vehicle controls ($P < 0.0001$).

On the other hand, MAL exposure induced a general decrease in GPx activity in almost all brain structures, especially in females (Fig. 6E). In the hippocampus, the two-way ANOVA revealed a significant effect of treatment ($F (1, 8) = 6.67$, $P = 0.032$) and the interaction treatment × sex ($F (1, 8) = 19.06$, $P = 0.024$). While Tukey's test showed a significant decrease only in female treated mice ($P = 0.051$). In the cerebellum, there was a clear tendency of the effect of treatment ($F (1, 8) = 3.68$, $P = 0.091$) and the interaction treatment × sex ($F (1, 8) = 18.95$, $P = 0.0024$). The post hoc test also revealed a significant decrease in GPx activity in only female-treated mice ($P = 0.0094$). As for the hypothalamus, there was an effect of both factors' treatment ($F (1, 8) = 41.41$, $P = 0.0002$) and sex ($F (1, 8) = 30.09$, $P = 0.0006$). The post hoc test demonstrated that GPx activity presented a clear tendency to decrease in treated females ($P = 0.058$), whereas it significantly decreased in treated males ($P = 0.0014$).

Discussion

In neurotoxicology, up to this day, there is so little known about the neurotoxicity of OPs and sex differences (Comfort and Re 2017). The purpose of this study is to investigate how MAL, an OP alters brain biochemicals and behavioral outcomes according to sex. The data presented here demonstrate that MAL chronic low-level exposure during a critical window of development induced sex-dependent neurotoxic effects, highlighted by a decrease in body weight and feeding behavior and several neurobehavioral deficits, accompanied by neurochemical alterations in different brain regions.

In our experiments, and because our focus is to study chronic low-dose exposure that represents the case of the majority of the world's population, no overt toxicity signs were observed in any of the treated groups. To assess pesticide toxicity, body weight is often used as a morphological marker. Several in vivo studies have found that organophosphate insecticides induce decreased body weight (Djekkoun et al. 2022; Lasram et al. 2014). In the present study, the body weight of male and female treated groups was significantly lower than their sex-matched vehicle controls. Besides, treated males presented a more drastic decrease in body weight (4 weeks onwards) than females (8 weeks onwards), suggesting sex- and time-dependent effects of MAL on body weight. Our findings are consistent with the results presented by other studies with a similar period of exposure to chlorpyrifos (CPF), which is another OP (Djekkoun et al. 2022; Reygner et al. 2016). It has been suggested

by many researchers that a decrease in body weight may be due to increased oxidative stress and enhanced catabolism of lipids and proteins (Djekkoun et al. 2022; Gupta et al. 2020). Decreased body weight might also be associated with decreased food consumption, which is the case in our study where both sexes demonstrated altered feeding behavior with a strong MAL response in male mice than in females, thus explaining their dramatic loss of body weight compared to females. Similar disturbances were declared in other experimental animals (Lasram et al. 2014; Seif et al. 2015). During the period evaluated both body weight and food intake in male mice were higher than that in females; these gender differences are knowingly explained by sex hormones (e.g., estrogens and testosterone) and sex chromosomes (Palmer and Clegg 2015; Chen et al. 2012). In addition to that, a recent study found that in the hypothalamus, female mice pro-opiomelanocortin (POMC) neurons display higher neural activities, compared to male counterparts, and further identified the transcription factor, TAp63, as one key molecule that contributes to the enhanced POMC neurons functions in females that allows them a certain protection from gaining weight (Wang et al. 2018). Indeed, the regulation of food intake requires the coordinated response of several central and peripheral factors. As mentioned earlier, the most studied central factor for the past two decades is the hypothalamic arcuate nucleus, where two neuronal populations are expressed, either POMC or neuropeptide Y and agouti-related peptide (NPY/AgRP). The peripheral factors are hormones including insulin, ghrelin, etc. (Williams and Elmquist 2012). In fact, it has been found that MAL increased plasma insulin levels in rats, and insulin is known to reduce food consumption by acting locally to inhibit hypothalamic NPY mRNA expression (Lasram et al. 2014; Schwartz et al. 2000). Moreover, research suggests that increased extracellular acetylcholine (ACh) in the nucleus accumbens (NAc) can inhibit feeding behavior. The cholinergic influence in the hypothalamus also plays a role in food intake regulation (Avena and Rada 2012). Actually, our results demonstrate decreased levels of AChE activity in the hypothalamus of treated mice, and highly more so in males than females, which promotes ACh levels in the hypothalamic synapses, thus food intake is attenuated especially in males. Furthermore, in the three weeks of food intake evaluation, both sexes displayed distinct food consumption patterns, where females showed an unstable and fluctuating profile and males presented a slightly steady curve throughout the experiment. Estrogen is well known to exert an inhibitory effect on feeding behavior (Olofsson et al. 2009). In female mammals, food intake is lower during the periovulatory period of the ovarian cycle, which coincides with a rise in serum estrogen levels (Asarian and Geary 2002). For instance, in rats, food intake decreases during estrus compared to metestrus and diestrus (Alonso-Caraballo and

Ferrario 2019); this may explain the fluctuations found in the food intake curve of female mice.

Following developmental exposures to OP insecticides, rodents have been found to exhibit a range of neurobehavioral impairments and oxidative stress, that are often sexually dimorphic, and depend on the dose and the exposure duration (Todd et al. 2020). In our study, long-term exposure to MAL extending from gestation to adulthood induced a decrease in locomotor activity in both sexes in the OF test, the same was reported in a previous study on rats (N'Go et al. 2013a). However, motor coordination was only altered in treated female mice in the Rotarod test, indicating a sexually dimorphic response. Another study also found that motor coordination was only impaired in female rats following a developmental exposure to CPF correlated with an increase of extracellular GABA in their cerebellum (Gómez-Giménez et al. 2018). The same was reported in a cohort study of infants showing signs of prenatal exposure to OPs (Silver et al. 2017). Moreover, It is well known that for brain development, ACh and cholinergic projection play a central role in synaptogenesis and the development of normal neural cytoarchitecture (Hohmann 2003). So, any interference of MAL with the cholinergic and dopaminergic system due to AChE inhibition would lead to several behavioral impairments, including motor development or motor coordination deficits (Ramos et al. 2006; Fereidounni and Dhawan 2018). The cerebellum is traditionally viewed as the structure that computes how to make movements smooth and coordinated (Manto et al. 2012). Hence, the significant decrease in AChE activity in the cerebellum of treated females and not in males is correlated with their decreased rotarod latency.

The present study also revealed that chronic exposure to MAL disrupts recognition memory in both sexes, as shown in the object recognition task where the treated groups exhibited a lower discrimination index compared to their control groups. On the other hand, short-term spatial working memory was significantly altered in treated female mice only, once again displaying a sexually dimorphic neurobehavioral impairment. Several studies have shown that MAL interferes with learning and memory function (N'Go et al. 2013b; Santos et al. 2016; N'go et al. 2021). Also, behavioral studies have long suggested that the hippocampus plays a crucial role in memory and learning (Squire 1992). However, unlike special memory, which relies heavily on hippocampal substrates, object recognition memory appears to rely on a variety of brain regions and the involvement of the hippocampus is unsettled (Denninger et al. 2018). Thus, in this study, we note that chronic low-level exposure to MAL was sufficient to cause a significant decrease of AChE in the hippocampus of female-treated mice only, which subsequently resulted in their marked learning and spatial memory impairments. Another plausible explanation for the general memory dysfunction, is the mitochondrial dysfunction,

astrogliosis, and activation of proapoptotic proteins in the hippocampus, as reported by Santos et al. (Santos et al. 2016) following repeated low-dose MAL exposure. There is growing evidence suggesting that Male rodents outperform females on a range of spatial learning tasks (Hawley et al. 2012; Leary et al. 2022; Yagi and Galea 2019). One of the reasons behind females' lower ability in spatial learning and memory is their lower magnitude of long-term potentiation (LTP), which is the main hippocampal mechanism that modulates spatial learning and memory (Bliss and Collingridge 1993; Morris and Frey 1997; Monfort et al. 2015; Safari et al. 2021). Taken together, these findings might further explain the sex difference found in spatial memory performance following MAL exposure.

Moreover, MAL-treated animals showed enhanced immobility time, an indication of depressant-like behavior in both sexes, but highly more so in females than males. Indeed, there is a positive association between depression and MAL exposure in male workers frequently exposed to this pesticide (Beard et al. 2014; Harrison and Mackenzie 2016), and despite females presenting over 40% of the global agricultural workforce, most studies are solely focused on male participants (Raney et al. 2011; Malekirad et al. 2013). Furthermore, several behavioral studies have also revealed depressive-like phenotypes following acute and chronic exposures to MAL, correlated with decreased AChE activity in some brain areas associated with depression (Saeedi Saravi et al. 2016; Savall et al. 2020; Ardebili Dorri et al. 2015; Pinto Savall et al. 2021). Our results are in line with these findings, and also reveal decreased levels of AChE activity, especially in the hippocampus of female treated mice, which is known to play a major role in depression (MacQueen and Frodl 2011). Interestingly, a study found that nitric oxide (NO) plays a role in the sex difference of depression-like behaviors in rodents, where female mice presented lower NO production in the hippocampus and were significantly more likely to display depressive behaviors than their male littermates, these behavioral sex gaps were mended when differences in the hippocampus NO levels were abolished between male and female mice (Hu et al. 2012).

Furthermore, our study demonstrates that MAL treatment results in a male-specific reduction in sociability, whereas females showed comparable sociability in the three-chamber test. Notably, autistic individuals are primarily characterized by deficits in social interaction and communication (Happé and Ronald 2008). Hence, the male-dependent effect of treatment on this social behavior is reminiscent of the strong male bias found in human autism spectrum disorder (ASD) prevalence (Dworzynski et al. 2012). On top of that, gestational OP exposure was associated with social deficits that were more prominent among boys than girls (Furlong et al. 2014). Similar to our results Lan et al. (Lan et al. 2019) also showed sex-selective

social deficits, where only males were impaired in the social preference test, following gestational exposure (GD 12-15) to a subtoxic dose of CPF (5 mg/kg). However, one of the limitations of this study is not taking the hormonal state of tested females into consideration, since sexual receptivity may be a factor that differentially regulates sociability (Jeon et al. 2018). Indeed, a recent study found that sexually-receptive (proestrus and estrus) mice showed no preferential interest in a novel female mouse compared with an empty chamber, while non-receptive (metestrus and diestrus) females showed normal preference similar to males (Chari et al. 2020). Nevertheless, our study is the first to carefully examine MAL exposure effect on males versus females in a non-receptive state and revealed sex-dependent social deficits. Additionally, our results showed that MAL treatment induced a male-specific increase in marble burying, which is a measure of repetitive and perseverative behaviors that represents some of the core behaviors in ASDs (Thomas 2009; Silverman et al. 2010). Raising the possibility that MAL exposure, one of the most used OPs around the world, might be a pervasive risk factor for ASD as von Ehrenstein et al. (Ehrenstein et al. 2019) also reported; thus more in-depth investigations are required.

Other than inhibiting AChE activity, OPs are also known to induce their neurotoxicity via oxidative stress. In light of this, we assessed the degree of oxidative damage imposed by MAL chronic exposure by evaluating the level of antioxidant enzyme activities (SOD, CAT, GPx) in parallel with MDA as the biomarker of lipid peroxidation (LPO) in all brain areas investigated. Several studies have found that MAL induces oxidative stress by showing increased levels of MDA and depletion of GSH, SOD, and CAT activities (Coban et al. 2014; Hazarika et al. 2003). Contrariwise, other MAL dosing regimens triggered the increase of CAT and SOD activities (Fortunato et al. 2006). Likewise, we found a general increase of SOD activity in all brain structures of both sexes, except the cerebellum of females and the hypothalamus of male treated groups. As for CAT activity, we also found increased levels in olfactory bulbs and hypothalamus of both treated animals, but with no differences in the hippocampus and the cerebellum. However, we found a general decrease in GPx activity in almost all brain structures, especially in females, which is consistent with the results of Maris et al. (Maris et al. 2010) where a trend of GPx activity inhibition was observed especially in females upon an acute MAL treatment. Therefore, since both increase and/or decrease in antioxidant enzyme activities are indicators of oxidative stress (Khan et al. 2017), the disrupted levels of oxidative stress markers in this study suggest a potential effect of reactive oxygen species (ROS) in neuronal damage which is expressed by the behavioral alterations induced by MAL exposure. Moreover, MAL induced an overall increase in MDA activity in all brain areas, especially in females. Similarly, Delgado et al. (Delgado et al. 2006) reported that MAL administration for 28 days resulted in increased MDA levels in the brain tissues of rats. In fact, the lipophilic nature of MAL may allow it to interfere with the cellular plasma membrane and cause increased LPO (Hazarika et al. 2003). Coupled with the greater vulnerability of the brain to oxidative stress due to its poor antioxidant defense system and high metabolic rate (Delgado et al. 2006), it may partly explain the rise of MDA production in the brain of MAL-treated mice.

Conclusion

To conclude, our work demonstrates that chronic low-level exposure to MAL during a critical period of development not only induced a range of neurobehavioral impairments that are often sex-dependent but also provoked oxidative stress and altered AChE activity at varying degrees between sexes in almost all brain structures. While there is a multitude of studies covering the adverse toxicity of OPs, limited research is available on their effects across sex. Our findings showed that treated females were more vulnerable regarding their special memory, motor coordination and were significantly more depressed, while only treated males' sociability was impaired and their food intake has highly plummeted. Nonetheless, more research is required to understand the mechanisms behind these sexually dimorphic responses in animal models, which may explain some of the patterns underlying the dimorphic susceptibilities noticed in neurological disorders.

Declarations

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