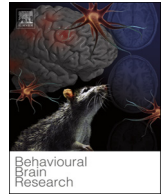




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Research report

Effects of lipopolysaccharide administration and maternal deprivation on anxiety and depressive symptoms in male and female Wistar rats: Neurobehavioral and biochemical assessments



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ABSTRACT

Introduction: Preclinical studies of early-life adversity (ELA¹) have highlighted the role of postnatal stress in the emergence and persistence of anxiety and depressive disorders. In this study, we compared anxious and depressive behaviors and oxidation levels in male and female Wistar rats subjected to three ELAs (lipopolysaccharide (LPS) induced, maternal deprivation (MD), or combination of the two stressors).

Methods: Rats were split into four groups: control group which received an intraperitoneal (IP) injection of saline on postnatal day (PND) 1, LPS-treated group which received an IP injection of LPS on PND1, MD group which was exposed to a 24-hour period of isolation on PND9, and LPS-treated/MD group which received an IP injection of LPS on PND1 then was exposed to a 24-hour period of isolation on PND9. Each group consisted of 12 rats and had an equal gender distribution. At three months, rats were subjected to neurobehavioral assessments and biochemical oxidative assays.

Results: Compared to controls, rats in the LPS and MD groups scored significantly higher on anxiety and depression-related measures. Gender differences in response were mainly observed in the MD group. Exposure to the combination of stressors led to a characteristic decrease in anxiety and an increase in depressive measures in both genders. All groups exposed to ELA showed a statistically significant increase in their oxidative stress levels.

Conclusion: Response to ELA is gender-dependent and modulated by the nature, type, and number of stressors. Further investigations are critical to understand the mechanisms underlying combination of stressors and gender's effect.

1. Introduction

Early life adversity (ELA), including childhood maltreatment, low socioeconomic status, and maladaptive family environments, has long-lasting effects on health outcomes across the lifespan [1,2]. This stems from a disturbance of major developmental processes of neurogenesis, synaptogenesis, and pruning in the critical period of childhood development, subsequently inducing enduring changes in brain development [3]. In particular, research describes a variety of ELA-mediated physiological disturbances, from cell-mediated inflammatory alterations (with elevation in markers of inflammation) [4,5], to neurohumoral changes (involving the hypothalamic–pituitary–adrenal axis and corticotropin-releasing factor neurotransmission) [6], and structural

variations (including an increase in the size of the amygdala and an attenuation in the development of the hippocampus (HP) and prefrontal cortex (PFC)) [7,8]. The ELA-mediated physiological disturbances “shape” a preexisting genetic vulnerability to disease into increased susceptibility to developing it. Indeed, ELA increases the risk of various psychopathologies at adulthood [9–11], including cardiovascular disorders [12,13], metabolic disturbances [14,15], oncological diseases [16], and mental illnesses [17,18]. In particular, accumulating evidence suggests that ELA constitutes a major risk factor for the emergence and persistence of psychiatric disorders, including anxiety, depression [15,19–21], schizophrenia [22–24], attention-deficit hyperactivity disorder, and posttraumatic stress disorder [25,26].

In line with this concept, animal models of rodents or nonhuman

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¹ Early-life adversity: ELA. Lipopolysaccharide: LPS. Maternal deprivation: MD. Intraperitoneal: IP. Postnatal day: PND. Phosphate buffered saline: PBS. Nitric oxide: NO. Prefrontal cortex: PFC. Hippocampus: HP. Thiobarbituric-acid-reacting substances: TBARS. Superoxide dismutase: SOD. Nitroblue tetrazolium: NBT.

primates have shown to be useful tools in improving our understanding of psychopathology post-ELA. Daily repeated removal of rat pups from their mothers, referred to as maternal deprivation (MD), is one relevant model of early life stress [27]. Models of postnatal inflammation [21,28,29] are also widely used paradigms involved in inducing anxiety and depressive behaviors in rodents. Studies using MD have highlighted the importance of ELA in the development of future depressive and anxiety disorders [30,31]. This is mediated by an alteration in several neurotransmitter systems, including dopaminergic, serotonergic [32–35], and glutamatergic activities [36]. MD in rats also negatively impacts the development of brain structures, including the HP [37,38] and neocortex [39]. It finally increases levels of inflammatory markers in adult brain areas (interleukins and tumor necrosis factor- α) [40,41], corticosterone levels [42], ACTH levels in response to stressors [43], and brain lipid peroxidation and superoxide dismutase (SOD) enzyme activity levels [44]. On the other hand, animal models of postnatal inflammation commonly use lipopolysaccharide (LPS), which administration triggers the release of inflammatory cytokines and the activation of microglia cells in the brain [45–47]. LPS administration in rodent pups results in increased depressive and anxiety-like behaviors [21,29,48–51], microglial activation [46], and tumor necrosis factor- α and interleukin-1 β levels in the HP of adult rats in response to stress [45]. In addition, neonatal LPS exposure causes persistent abnormalities in the hypothalamic–pituitary–adrenal axis function [29,46,48–50] with elevation in corticosterone levels [50].

However, most previous preclinical studies strictly use male rodents or, when examining the effect of gender on the impact of ELA, often show varied results [34,44,52–55]. This becomes of relevance as the relationship between gender, ELA, and the emergence of psychiatric disorders is complex. This poorly studied intricate relation stems from gender differences in the nature of ELA, sexually dimorphic effects of ELA on brain development, and gender variations in brain laterality [7]. Although a large number of studies have evaluated the effect of ELA on long-term depression and anxiety-related behaviors, the role of gender in the equation remains poorly understood. In addition, to our knowledge, most studies used maternal deprivation as ELA-model with less focus on infectious models, or on the combination of these two etiologically-different stressors. Therefore, in our study, we compared behavioral differences between Wistar rats under each of three groups of ELA (LPS-induced, MD, and the combination of the two stressors) and behavioral differences between genders, and further analyzed individual differences.

2. Methods

2.1. Animal model

Male and female Wistar rat pups were used for the experiment. Initially, pregnant female Wistar rats ($n = 10$) provided by the laboratory of Genetics, Neuroendocrinology, and Biotechnology of Ibn Tofail University were individually housed in standard plexiglass cages ($430 \times 290 \times 210$ mm) at a constant temperature of 24°C , with a relative humidity of 50–60%. A 12-hour light-dark cycle was maintained with lights turned on from 19:00 pm to 7:00 am along with unlimited access to both food and water. The pregnant females yielded a total of 80 pups (30 males and 50 females). Newborn pups were checked daily at 9:30 am. If a Wistar rat pup was born, its day of birth was defined as postnatal day (PND) 0.

5 pups were born dead. On the day after parturition, the remaining living pups were randomly allocated to one out of four groups, described in Section 2.2. Taking into consideration the mortality rate throughout the course of the experiments ($n = 15$ or 20% of the living pups – all of which died after LPS injection), each group was catered to have a total of 12 healthy pups with an equal male to female ratio. From the total living yield of litters, 12 pups remained at the end of the allocation and were donated for other research groups.

The tail of each randomized rat was marked according to the group to which it belonged. Pups from the same group and gender were placed in one plexiglass cage (total of 8 cages). Then, 8 of the adult mothers were randomly assigned to the cages while the remaining 2 mothers were donated for other research groups.

This work has been fully realized in the Genetics, Neuroendocrinology, and Biotechnology laboratory located at Ibn Tofail University in Kenitra, Morocco. All experimental procedures were performed according to the National Institutes of Health guide for the care and use of Laboratory animals and were authorized by the Doctoral Study Center at the university.

2.2. Experimental treatments

Male and female Wistar rat pups were randomly assigned to one out of four groups. Each group consisted of 12 pups with an equal gender distribution (6 males and 6 females):

- **Control group:** pups were administered intraperitoneal (IP) injection of phosphate buffered saline (PBS) (0.1 mg/kg) on PND1 without MD
- **LPS-treated group:** pups remained undisturbed with their families and then administered an IP injection of LPS [*Escherichia coli*, serotype 026: B6; L-3755 (Sigma, St. Louis MO); $250\ \mu\text{g}/\text{kg}$] on PND1
- **MD group:** pups were exposed to an individual period of isolation, for 24 h, on PND9
- **LPS-treated/MD group (LPS/MD group):** pups were administered an IP injection of LPS on PND1 and then exposed to an individual period of isolation, for 24 h, on PND9.

At 21 days of age, all male and female rat pups were weaned and separated from their mothers. Animals were housed in their respective cages until 90–97 days of age when long-term effects of early life adversity were assessed. We used the following behavioral tests: open field test, novelty suppressed feeding test, elevated plus maze test, black-white box test, and forced swimming test. We analyzed the performance of the rats on these protocols and accordingly measured their levels of anxiety and depression. Following the behavioral assessments, we performed biochemical analyses of central and peripheral tissues in order to assess nitric oxide level, lipid peroxidation, and superoxide dismutase activity. A schematic representation of the design of the study is presented in Fig. 1.

2.3. Behavioral tests

All behavioral tests were carried out between 90 and 97 days of age. To assess anxious and depressive behaviors, all animals were exposed to the five previously mentioned tests. Before the application of each assessment, the rats were placed in the testing apparatus under appropriate illumination. All behaviors were then recorded for subsequent analysis.

2.3.1. Open field test

To evaluate anxiety and locomotion measures, we used an open-field test. The field consists of a wooden made apparatus (100×100 cm) enclosed by a 40 cm height wall and placed under strong illumination (100 W, 2 m above the apparatus). The area is divided into 25 squares (20×20 cm), defined as 9 central and 16 peripheral squares. At the beginning of the test, each animal is placed in the center of the apparatus and then allowed to freely explore it for 10 min. In between testing, the apparatus is cleaned using 70% ethyl alcohol.

The quantified parameters were the number of total squares visited (a measure of locomotor activity), the time spent in the central area (a measure of anxiety) [56], and the number of returns to the 9 central

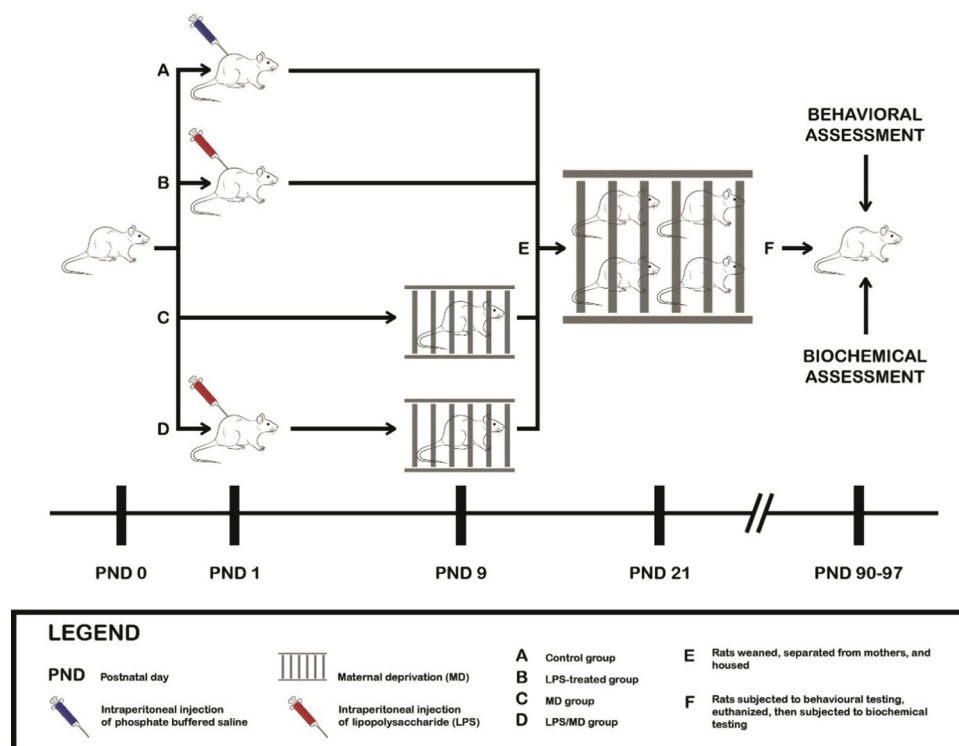


Fig. 1. Schematic representation of the design of the study and timeline of the conducted experiments.

squares (a measure of anxiety) [57]. The central area of a novel environment is anxiogenic and aversive; therefore, the behavioral inhibition appears as an avoidance towards the central zone of the open field [58].

2.3.2. Novelty suppressed feeding test

We carried the novelty suppressed feeding test to further assess for anxiety. The test is done in an open field set up under bright lighting conditions. In this test, rats are subjected to food restriction for a period of 24 h. After food restriction, the rats are placed in the open field, next to a pellet of food located at the center of the arena. The anxiety-related measure is the recorded amount of time needed to approach and to eat the food.

2.3.3. Elevated plus maze test

We proceeded with an elevated plus maze test to evaluate anxiety-like behaviors. The test is made of two wooden open arms (50 × 10 cm) crossed at right angles with two opposed arms of equal sizes. Two of the opposed arms are enclosed by wooden walls of 40 cm in height, except at the central part where the arms cross. The whole apparatus is elevated 50 cm above the floor. For the test, each animal is placed at the center of the plus maze, facing one of the closed arms, and allowed to freely explore the arena for 5 min. The anxiety-related measures include the time spent by a rat in the open arms and the duration of stretching in the closed arms. In between testing, the apparatus is cleaned using 70% ethyl alcohol [59].

2.3.4. Black-white box test

The black-white box (BWB) test allows the evaluation of an animal's aversive reaction in a highly illuminated situation [60]. The apparatus consists of two compartments (25 × 25 × 25 cm): one is dark (black chamber) whereas the other (white chamber) is highly illuminated (1700 lx). The compartments are connected via a tunnel (7 × 7 × 10 cm). Animals are individually placed on the dark side. The time spent in the white chamber, the latency to enter into the lighted area, and the number of dark-light transitions are measured for 6 min as

an index of anxiety.

2.3.5. Forced swim test

We assessed depressive mood symptoms using a forced swim test. In this test, swimming sessions are conducted by placing the rat in individual glass cylinders (height = 50 cm; diameter = 30 cm) containing 30 cm of water at a temperature of $23 \pm 2^\circ\text{C}$. During a session, rats are forced to swim in the cylinder for 5 min and their duration of immobility is recorded. A rat is judged immobile when it ceases all active behaviors (i.e. struggling, swimming, and jumping) and remains passively floating, making a minimal movement to maintain its nostrils above water. The latency to the first bout of immobility is also recorded. Increased time of immobility is interpreted as an increased depressive-like response (measure of depression) [61].

2.4. Biochemical analyses

2.4.1. Tissue preparation

After the behavioral tests, all animals were deeply anesthetized with chloral solution (100 mg/kg). The PFC and HP of each hemisphere were separately dissected and homogenized using Dounce homogenizer in an ice-cold lysis buffer (RIPA lysate solution + 1 mM PMSF). The homogenates were centrifuged for 15 min (14,000 g) then stored at -80°C . The liver and spleen tissues were similarly prepared.

2.4.2. Nitrite/nitrate assay

In the aqueous solutions of biological systems, the conversion of nitric oxide (NO) to nitrite and nitrate is thought to favor nitrite production. Nitrite is the only stable end-product of the auto-oxidation of NO; therefore, measurement of its concentrations in the serum and tissue homogenates is widely accepted as an index of NO activity [62].

In this study, we measured concentrations of nitrite in the rats' brain tissue homogenates (PFC and HP) by using the diazotization method based on the Griess reaction, an indirect assay for NO production [63]. Samples of tissue (500 μl) were pipetted into tubes and an equal volume of Griess reagent (1% sulphanilamide (1 ml) and 0.1% N-1-naphthyl

ethylenediamine dihydrochloride (1 ml) in 2.5% orthophosphoric acid) was then added in each tube. After incubation for 30 min at room temperature, absorbance was measured at 540 nm. Linear regression analysis was used to calculate the nitrite concentrations in the sera and tissue homogenates using the standard calibration curves of sodium nitrite. Tissue nitrite levels were expressed in mmol/g protein.

2.4.3. Lipid peroxidation assay

The formation of lipid peroxides in the PFC and HP was analyzed by measuring the level of thiobarbituric-acid-reacting substances (TBARS) in cells [64]. The samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% and heated for 15 min in a boiling water bath (90 °C). Butanol (2:1 v/v) was then added to the solution. After centrifugation for 5 min (8000 g), the level of TBARS was determined by the absorbance at 535 nm [65]. Similarly, for the liver and spleen tissues, TBARS measurement was done by mixing the tissue homogenates with 1 ml of trichloroacetic acid 10% and then heating it for 60 min in the boiling water bath [66]. The lipid peroxidation concentration was expressed as $\mu\text{mol/g}$ tissue.

2.4.4. Superoxide dismutase assay

The SOD activity was determined according to the protocol described by Beauchamp and Fridovich [67]: Illumination of riboflavin in the presence of oxygen and electron donors, such as methionine, generates superoxide anions which inhibits nitroblue tetrazolium (NBT) reduction.

The 1 ml reaction mixture consisted of 0.94 ml of 50 mM phosphate buffer (containing 12 mM methionine, 75 μM NBT, 0.1 mM EDTA, 0.025% Triton X-100 and 2 μM riboflavin, with pH 7.4) and 0.06 ml of supernatant. The SOD assay was carried by placing the mixture in yellow light for 10 min. A control mixture without the enzyme source was also included. The reduction of NBT by superoxide radicals to blue-colored formazan was determined by the absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme needed to inhibit the reduction of NBT by 50% under the above conditions [67]. This activity was expressed as U/g of brain tissue.

2.5. Data analysis

All data were analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for comparison between groups. All tests were used with significance set at $p < 0.05$. The data were presented as mean \pm standard error of the mean.

3. Results

3.1. Measures of anxiety

Our results showed comparable outcomes for the different behavioral tests assessing anxiety measures in Wistar rats exposed to early

adversity. In the open field test, as shown in Fig. 2A, both males and females in the LPS, MD, and LPS/MD groups had a statistically significant increase in the total number of squares they visited compared to their respective controls ($p < 0.001$ except for the female LPS/MD group with $0.001 < p < 0.01$). No gender differences were noted, except for females in the MD group which exhibited a significantly higher total number of visited squares compared to their male counterparts ($0.001 < p < 0.01$) (Fig. 2A).

The number of revisited central squares was the main measure of anxiety in the open field test. As shown in Fig. 2B, there was a statistically significant decrease in the number of revisited central squares in the male MD group compared to controls ($0.01 < p < 0.05$). This stood in contrast to a pattern of increase in the number of revisited squares in the male LPS/MD ($0.001 < p < 0.01$), female LPS ($p < 0.001$), and female LPS/MD ($0.001 < p < 0.01$) groups. A statistically significant gender difference was noticed amongst groups: females exhibited more revisits compared to their male counterparts ($p < 0.001$ for LPS and MD groups, $0.001 < p < 0.01$ for LPS/MD group) (Fig. 2B). In addition, only males in the MD group spent significantly less time in the central area of the open field compared to controls ($p < 0.001$). In contrast, LPS and LPS/MD female groups spent significantly more time in the central area than controls ($p < 0.001$) (Fig. 2C). A gender difference was noticed between the various groups, with less time spent by all females compared to their male counterpart Wistar rats ($p < 0.001$).

On the other hand, LPS males showed a statistically significant decrease in the number of entries in the open arms of the elevated plus maze test compared to controls ($0.001 < p < 0.01$). A comparable but less significant trend was observed in both LPS and MD female groups ($0.01 < p < 0.05$) (Fig. 3A). Likewise, as presented in Fig. 3B, both genders spent significantly less time in the open arms across the LPS ($p < 0.001$), MD ($p < 0.001$), and LPS/MD ($p < 0.001$ for males and $0.001 < p < 0.01$ for females) groups. No gender differences were noted between groups in the elevated plus maze test.

In the novelty suppressed feeding test, male and female rats in the LPS and MD groups exhibited an increase in their latency feeding time compared to controls ($p < 0.001$). Noticeably, no difference was observed between LPS/MD males or females and their respective controls. The latency feeding time was significantly shorter only in females in the MD groups compared to their male counterparts ($0.01 < p < 0.05$) (Fig. 4).

Finally, in the BWB test, males and females in the ELA groups exhibited a statistically significant decrease in the time they spent in the white chamber compared to controls ($p < 0.001$ for LPS and MD groups, $0.01 < p < 0.05$ for LPS/MD group) (Fig. 5A).

Males in only LPS and MD groups had a significant decrease in the number of entries in the white chamber ($0.01 < p < 0.05$ and $0.001 < p < 0.01$, respectively) and a significant increase in their spent time in the black chamber ($p < 0.001$) compared to controls (Fig. 5B and C). Similarly, females in the LPS and MD groups showed a

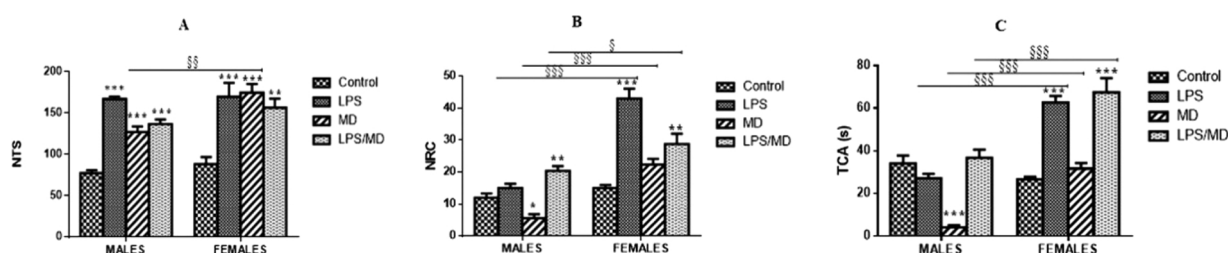


Fig. 2. Behavioral performances of male and female rats in the open field test. A. Number of total squares (NTS) visited. B. Number of returns into center (NRC) and revisited central squares. C. Total amount of time spent in the central area (TCA), measured in seconds.

The results are represented as means \pm the mean standard error (SEM). The endings of the bars indicate the compared groups that have a statistically significant difference.

The symbols * and § denote significant differences compared to control or to gender counterparts, respectively.

The significance level is 0.05 with (*, §) $0.01 < p < 0.05$, (**, §§) $0.001 < p < 0.01$, and (***, §§§) $p < 0.001$.

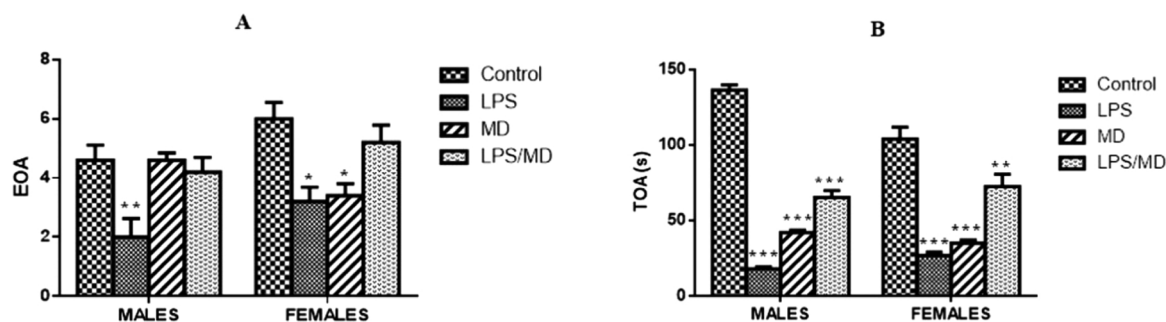


Fig. 3. Behavioral performance of male and female rats measured in the elevated plus maze test. A. Number of entries in the open arms (EOA). B. Total amount of time spent in the open arms (TOA), measured in seconds.

The results are represented as means ± the mean standard error (SEM). The endings of the bars indicate the compared groups that have a statistically significant difference.

The symbols * and § denote significant differences compared to control or to gender counterparts, respectively.

The significance level is 0.05 with (*, §) 0.01 < p < 0.05, (**, §§) 0.001 < p < 0.01, and (***, §§§) p < 0.001.

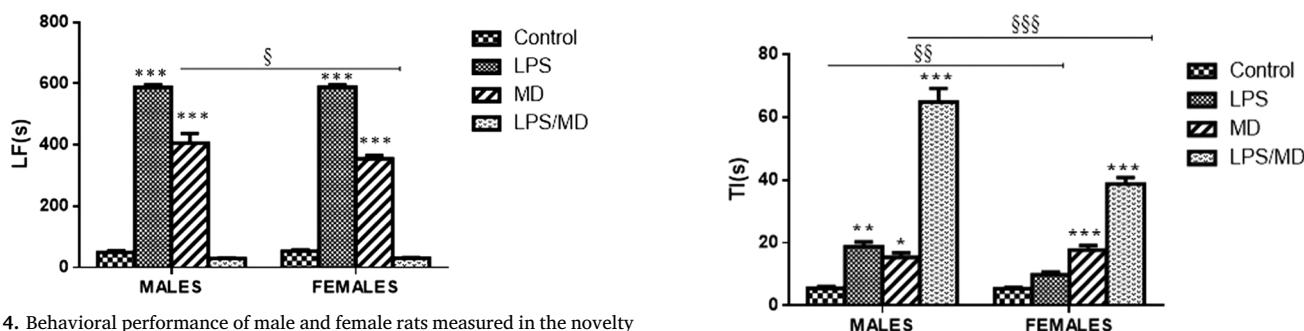


Fig. 4. Behavioral performance of male and female rats measured in the novelty suppressed feeding test. Total amount of latency for feeding (LF), measured in seconds.

The results are represented as means ± the mean standard error (SEM). The endings of the bars indicate the compared groups that have a statistically significant difference.

The symbols * and § denote significant differences compared to control or to gender counterparts, respectively.

The significance level is 0.05 with (*, §) 0.01 < p < 0.05, (**, §§) 0.001 < p < 0.01, and (***, §§§) p < 0.001.

significant decrease in their number of entries in the white chamber (p < 0.001) (Fig. 5B) with a prolonged total time spend in the black chamber (p < 0.001 for the MD group and 0.001 < p < 0.01 for the LPS group) (Fig. 5C). No difference was observed between the LPS/MD group and controls for these 2 parameters. No gender differences were noted between groups for all parameters in the BWB test.

3.2. Measures of depression

The time of immobility in the forced swimming test was used to assess for depression. A general pattern of prolonged immobility was observed in male MD (0.001 < p < 0.01), male LPS (0.01 < p < 0.05), male LPS/MD, female MD, and female LPS/MD (p < 0.001) groups compared to controls (Fig. 6). Gender differences

Fig. 6. Behavioral performance of male and female rats measured in the forced swimming test. Total time of immobility (TI), measured in seconds.

The results are represented as means ± the mean standard error (SEM). The endings of the bars indicate the compared groups that have a statistically significant difference.

The symbols * and § denote significant differences compared to control or to gender counterparts, respectively.

The significance level is 0.05 with (*, §) 0.01 < p < 0.05, (**, §§) 0.001 < p < 0.01, and (***, §§§) p < 0.001.

were observed in the LPS (0.001 < p < 0.01) and LPS/MD groups only (p < 0.001), with more prolonged immobility in males.

3.3. Results of biochemical analyses

3.3.1. Level of nitric oxide in the prefrontal cortex and Hippocampus

The measurement of nitrite concentrations in the rats' brain tissue homogenates detected a statistically significant increase in NO levels in the PFC of males in the LPS (0.001 < p < 0.01), MD (p < 0.001), and LPS/MD groups (p < 0.001) compared to controls (Fig. 7A). A similar trend was also observed in the HP of male rats across the three groups (p < 0.001) (Fig. 7D). Likewise, in comparison to controls, female rats exposed to stress displayed a significant increase in their NO levels in both the PFC and HP (p < 0.001) (Fig. 7A and D). Gender

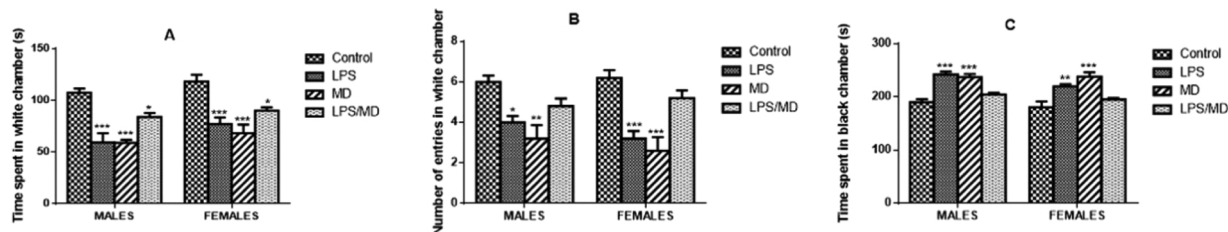


Fig. 5. Behavioral performance of male and female rats measured in the black-white box test. A. Total amount of time spent in the white chamber (TWC), measured in seconds. B. Number of entries in the white chamber (EWC). C. Total amount of time spent in the black chamber, measured in seconds.

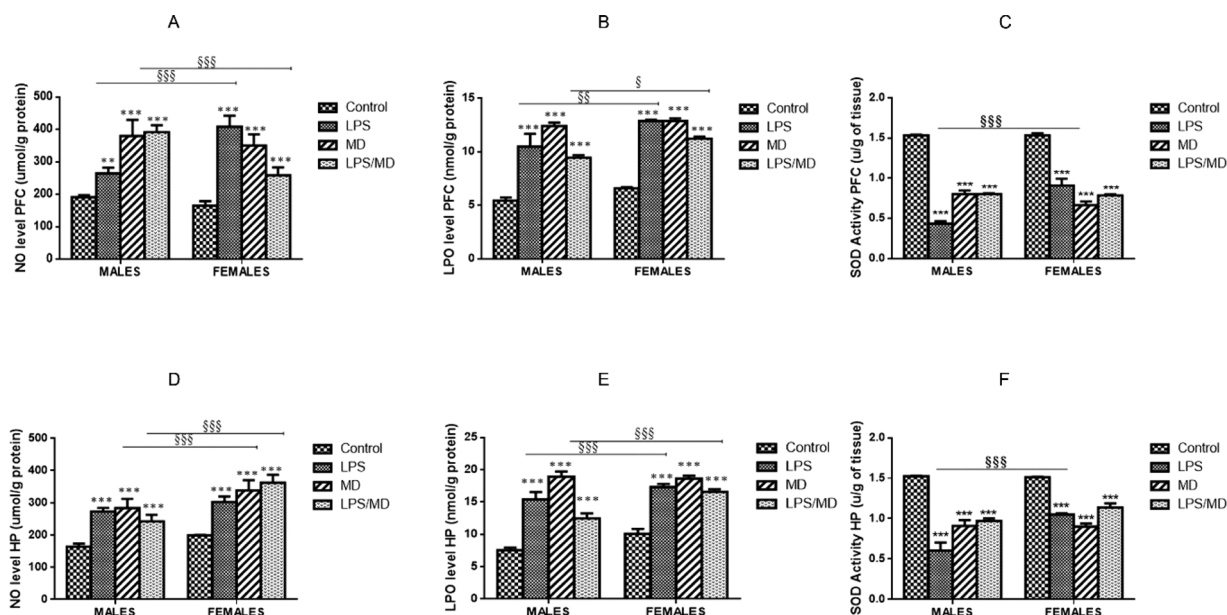


Fig. 7. Detection of nitric oxide (NO) level by Griess reaction, lipid peroxidation (LPO) level by TBARS, and superoxide dismutase (SOD) activity in the prefrontal cortex (PFC) and hippocampus (HP) of male and female rats. A, B, and C. NO level, LPO level, and SOD activity in the PFC, respectively. D, E, and F. NO level, LPO level, and SOD activity in the HP, respectively. NO and LPO levels are expressed in $\mu\text{mol/g}$ of protein. SOD activity is expressed in U/g of tissue. The results are represented as means \pm the mean standard error (SEM). The endings of the bars indicate the compared groups that have a statistically significant difference.

The symbols * and § denote significant differences compared to control or to gender counterparts, respectively.

The significance level is 0.05 with (*, §) $0.01 < p < 0.05$, (**, §§) $0.001 < p < 0.01$, and (***, §§§) $p < 0.001$.

differences were noted between groups; in particular, females in the LPS, and those in MD and LPS/MD groups displayed significantly higher NO levels compared to their male counterparts in the PFC and HP respectively ($p < 0.001$).

3.3.2. Level of lipid peroxidation in the prefrontal cortex and Hippocampus

Both males and females in the LPS, MD, and LPS/MD groups exhibited a statistically significant increase in their lipid peroxidation level compared to controls ($p < 0.001$). This was observed in both the PFC (Fig. 7B) and HP (Fig. 7E). Again, gender differences were noted between groups. In particular, females in the LPS and LPS/MD groups displayed higher peroxidation levels compared to their male counterparts. This was also observed in both the PFC ($p < 0.001$ for LPS group and $0.001 < p < 0.01$ for LPS/MD group) and HP ($p < 0.001$ for both groups).

3.3.3. Level of superoxide dismutase activity in the prefrontal cortex and Hippocampus

Rats of both genders in the three groups (LPS, MD, and LPS/MD) displayed a statistically significant decrease in their SOD activity in the PFC (Fig. 7C) and HP (Fig. 7F) compared to controls ($p < 0.001$). The SOD activity was gender-dependent only in the LPS group: males showed a significantly lower activity compared to females in both brain regions.

3.3.4. Level of lipid peroxidation in the liver and spleen

Compared to controls, males and females in the LPS, MD, and LPS/MD groups displayed a statistically significant increase in their level of lipid peroxidation in both the liver (Fig. 8A) and spleen (Fig. 8B) ($p < 0.001$). Gender differences were only noted for the oxidation level in the liver of the LPS/MD group, with males displaying a higher level of lipid peroxidation compared to their female counterparts ($0.001 < p < 0.01$).

4. Discussion

In this study, we assessed the anxiety- and depressive-like behaviors following ELA in male and female adult Wistar rats. The animals were exposed to either one or a combination of stressors. We then subjected them to a series of behavioral (open field, elevated plus maze, novelty suppressed feeding, black-white box, and forced swimming tests) and biochemical (nitrite/nitrate, lipid peroxidation, and SOD assays) tests. We found that, as a trend, ELA generates an anxiogenic and depressive effect in both males and females compared to controls. Gender differences in response were observed for most measures, particularly in the MD group. To our knowledge, no studies have previously investigated the effect of combined LPS administration and MD, as employed in our methodology, on anxiety and depressive parameters. Our results showed an anxiolytic but depressive response in this group compared to those exposed to a single stressor.

4.1. Measures of anxiety

When assessing anxiety measures, the open field, elevated plus maze, novelty suppressed feeding, and black-white box tests showed comparable results. In the open field test, only males in the MD group were more anxious compared to controls. Females across all groups were less anxious than their male counterparts. In the elevated plus maze, novelty suppressed feeding, and black-white box tests, both genders exposed to one adversity displayed more anxious behaviors compared to controls. Yet, no gender differences were found between groups.

Gender dimorphism in anxiety response to ELA has been previously described: in contrast to adult females, males commonly display an increase in anxiety-like and fear-associated behaviors following MD [68–71]. On the other hand, the effect of LPS injection is highly dependent on its dose and time of administration. To our knowledge, this is the first study assessing the outcome of $250 \mu\text{g/kg}$ of LPS provided at PND1. Other studies conducted in our lab and using the same dose describe an increase in anxiety-like behaviors in male rats compared to

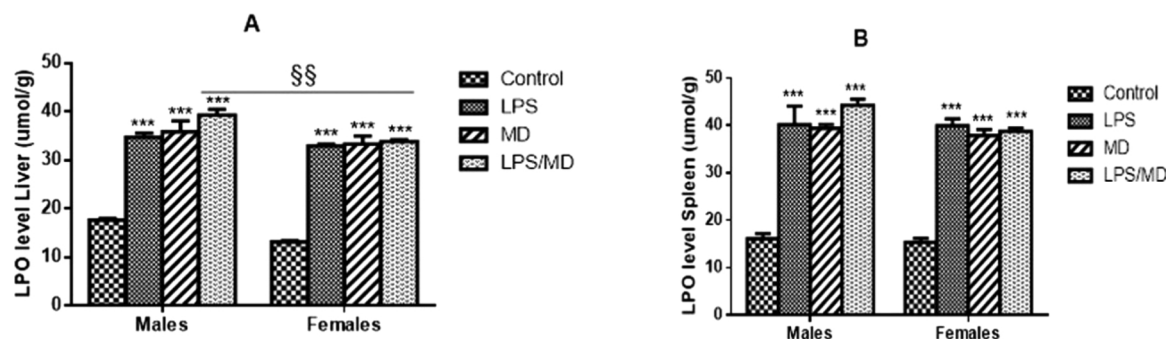


Fig. 8. Detection of lipid peroxidation (LPO) level by TBARS in the liver and spleen of male and female rats. A. LPO level in the liver. B. LPO level in the spleen. Levels are expressed in $\mu\text{mol/g}$ of protein.

The results are represented as means \pm the mean standard error (SEM). The endings of the bars indicate the compared groups that have a statistically significant difference.

The symbols * and § denote significant differences compared to control or to gender counterparts, respectively.

The significance level is 0.05 with (*, §) $0.01 < p < 0.05$, (**, §§) $0.001 < p < 0.01$, and (***, §§§) $p < 0.001$.

controls when LPS is provided at PND9 [72] and PND14 [73]. This comes in direct opposition to the findings of another recent study showing a decrease in anxiety and depression-like behaviors in males but no change in females, as compared to controls, after administration of 250 $\mu\text{g/kg}$ of LPS at PND14 [74].

Reports employing higher LPS doses also describe paradoxical results: there is an increase in anxiety-like behaviors in males injected with 830 $\mu\text{g/kg}$ of LPS at PND14 [75] in contrast to a decrease in such behaviors in female rats given 1 mg/kg at PND5 [76], as compared to their respective controls.

More consensus is nevertheless observed for research employing lower doses of LPS. Indeed, studies indicate that neonatal exposure to 50 $\mu\text{g/kg}$ of LPS at PND2 increases levels of anxiety in females [77] and, at PND3 and PND5, anxiety-like behaviors in male rats [50], in both genders without difference [48], or in males more than females [78,79] with a decrease in anxiety in females [79]. Alternatively, one report did not show any significant changes in levels of anxiety following the administration of 100 $\mu\text{g/kg}$ of LPS at PND 14 in male rats [80]. This was consistent with a more recent study under the same condition, showing no effect on anxiety-like behaviors in adult males but increased anxiety in females [81].

All of these data provide strong evidence of the sexual dimorphism in the effect of postnatal LPS exposure. The selective gender effect on ELA in general can be partially explained by the female cycle profile, as rats in the pro-estrous and estrous phases of their cycle display less anxiety-like behaviors compared to males [82–85]. This difference might also stem from selective dendritic hypertrophy and an increase of spine numbers in the basolateral amygdala of male rodents observed after MD [69]. One inference to be drawn from these discrepant findings is that effects on anxiety may be reliant on several factors, including age and strain of rat, severity, nature, and intensity of the ELA (i.e. the dose and time of administration of LPS), and behavioral tests, their sensitivity and specificity, and their period of evaluation.

4.2. Measures of depression

The results of the forced swimming test show a general pattern of increased depressive-like behaviors in males and females subjected to ELA compared to controls. This pattern was more substantial in males.

Our results go in harmony with previous literature reporting some form of depressive behavior in male rats exposed to MD or other stressors [53,86,87]. The dimorphic effect of gender on depressive response post-ELA has been also previously described, with prior pre-clinical studies highlighting the importance of gender factors in understanding individual variations in stress reactivity [88,89]. In addition, as stress circuits are strongly gender-dependent [90], ELA

may differentially impair males and females when tested with different behavioral tools during adolescence and adulthood [91]. In fact, while some data show a comparable increase in depressive-like behaviors in both males and females after MD [31], others show that female rats generally exhibit lower susceptibility to stress-induced depression following such event [92–95]. This has been recently reiterated in a review of preclinical studies, despite the large variation in the applied animal models, in terms of species and age of testing, and the measured outcome parameters [92].

The resilience of female rats to depressive outcomes after ELA can be accounted, on a neuropathophysiological level, to a maintained morphology of their neurons as early as one week after stress, at which time their neurons resemble those of unstressed rats and display significant increase in stubby spine density [96] and dendritic hypertrophy [97] compared to males. Estrogen was as well postulated to lower vulnerability to stress-induced depression in female rodents. Estrogen upregulates dopamine receptors [98,99]; its downregulation, therefore, plays a crucial role in the development of depression [100]. Along the same lines, female hormones protect ovariectomized animal models in tests of depressive-like behaviors [101,102]. These findings can explain why female rats are less sensitive to ELA than males when measuring mood-associated outcomes.

4.3. The combined stress model

The LPS/MD male and female groups had a tendency towards a more depressive, but interestingly, less anxious response compared to other groups in all behavioral tests. The exacerbation of depressive behaviors can be explained by the pathophysiology of the underlying condition. Based on the diathesis-stress theory, depression is a consequence of the cumulative effect of two or more stressors experienced over the lifespan [103,104]. In other terms, a primary adverse event programs the neurological system to increased vulnerability for future psychopathology upon exposure to additional stressors [105,106]. As such, the combination of ELAs in our animal model can account for the potentiation of the measured depressive outcomes.

The unexpected results of anxiety measures are also original as no previous report assessed the effect of LPS and MD combination on behavioral outcomes. At a biological level, a very recent study found that male rat pups subjected to MD and 1 mg/kg of LPS at PND14 displayed maximal activation of microglial cells and reduction of hippocampal astrocyte density, as compared to MD and LPS only groups. Yet remarkably, the MD/LPS rats demonstrated an attenuation in the secretion of their hippocampal interleukin-1 β and peripheral cytokines (interleukin-1 β , interleukin-6, and tumor necrosis factor- α) [107]. This inflammatory pattern is concordant with studies of adult mice exposed

to chronic stress during adolescence [108]. The severe central inflammatory state associated with the blunted peripheral immune activity could be an adaptive response to counteract the effect of a new stressor in individuals already compromised by exposure to primary adversity [107]. On a behavioral level, this might reflect as a decrease in anxiety-like behaviors. On another note, our results can be paralleled to other experiments employing different models of LPS administration, including postnatal [74,76,79] and prenatal injection, that demonstrate a possible anxiolytic effect on behavior. In one study using prenatal LPS exposure, this induced a significant reduction in the baseline anxiety levels of adult male mice, an effect abolished after administration of restraint stress or tumor necrosis factor- α [109]. This is possibly mediated by an indirect augmentation of GABAergic activity [110,111] or a secondary hippocampal injury and inflammation [76,112–114], both processes being linked with anxiolytic-like responses. Nonetheless, due to variations in the methodology of LPS administration between studies, the effects of such ELA may not be consistent throughout all reports. This may possibly account for the results obtained in our LPS/MD group.

4.4. Biochemical analyses

Compared to controls, all groups exposed to ELA showed a statistically significant increase in their levels of oxidative stress both centrally (in the PFC and HP) and peripherally (in the liver and spleen). Females had a trend towards more oxidative stress compared to their male counterparts, yet an overall comparable level. This is complemented by an overall decrease in SOD level.

Previous studies have shown that, in a model of repeated MD, there is increased lipid peroxidation [44,115] and reduced SOD activity in the HP, PFC, and striatum of male rodents, compared to more stable parameters in female rats [44]. This again related to the protective effect of female hormones, possibly through a scavenging action of estrogens [116]. Early-life adversity has also been associated with elevated inflammatory markers in the liver and spleen [117]. The increase in oxidation levels is concordant with previous studies of animal models describing a general downregulation of antioxidant defenses and elevated oxidative stress as molecular underpinnings for the development of depression and anxiety (see review [118]).

5. Limitations

Our study has several limitations. First of all, the small number of rats in each group might have skewed our results and decreased the power of the findings. Using the Bonferroni's post hoc test in our analysis has accounted for this drawback.

We are also aware that two major culprits in ELA, inflammation and epigenetic changes, have not been thoroughly assessed in this study as they were beyond the current scope. As LPS administration can cause cellular inflammation and as maternal deprivation might trigger epigenetic modifications, assessing these variables becomes necessary for a better understanding of the subsequently observed behavioral changes. We are currently undergoing further analyses of the brain samples of our rodents, including histology staining, assessment of inflammation (via tumor necrosis factor- α assay and immunohistochemistry), and epigenetic profiling (DNA and RNA analysis). This will be also complemented by results of cognitive testing done in the different animal groups.

Another limitation, yet a gateway for future research, relates to our model of LPS administration. As different study protocols employ LPS of different dosages (usually lower than 250 μ g) with variable timings of injection (commonly after PND1), consensus of LPS effect on neuropsychiatric sequela remains unclear. As part of our ongoing research, we are currently analyzing the effect of different dosages of LPS administration at variable postnatal days on neurobehavioral and biochemical assessments.

Finally, measurement of estrous hormonal levels would have provided more insight into the differential gender response observed in some of the behavioral tests and strengthened the estrogen hypothesis in regulating anxiety and depressive symptoms.

6. Conclusion

In conclusion, we provide evidence for a gender-dependent response in anxiety and depressive measures after ELA. Sexual dimorphisms are present in response to early life stress. Our results reiterate such differences through the variation in behavioral outcomes. Response is also modulated by the nature, type, and number of stressors. A combination of LPS administration and MD exacerbates depressive outcomes yet decreases those related to anxiety. As this is the first study portraying such results, the generalizability of our findings should be considered. Also, as cumulative load from simultaneous ELA of different natures has not been extensively tested in basic research, further investigation and design of adequate animal models is critical to understand the neurobiological mechanisms underlying early double insult, its relation to gender, and its subsequent neuropsychiatric consequences. As previously mentioned, we are currently assessing differences in the inflammatory and epigenetic states of our male and female rats post-ELA. This will be complemented by gender-based differential analysis of cognitive capacities following adversity.

Declarations of interest

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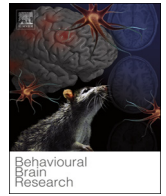


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Corrigendum

Corrigendum to “Effects of lipopolysaccharide administration and maternal deprivation on anxiety and depressive symptoms in male and female Wistar rats: Neurobehavioral and biochemical assessments” [Behav. Brain Res. 362 (2019) 46–55]



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The authors regret that authorship detail was incorrect in the article. The correct order is as follows:

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