



Impact of Early Immune Challenge by LPS on Nitric Oxide Release Dynamics in the Neonatal Rat Brain: Triggering Long-lasting Sex- and Age-Related Affective Behavioral Alterations in Adolescence

LAILA IBOUZINE-DINE^{1*}, INSSAF BERKIKS^{1,2}, HASNAA MALLOUK¹, MOULOUD LAMTAI¹, ZINEB EL MARZOUKI¹, AMAL DIMAOUI¹, ABDELHALEM MESFIOUI¹, ABOUBAKER EL HESSNI¹

¹Laboratory of Biology and Health, Neurosciences, Neuroimmunology and Behaviour Unit, Faculty of Science Ibn Tofail University, Kenitra, Morocco; ²Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham Heersink School of Medicine, Birmingham, USA.

Abstract | Early-life immune challenges, like lipopolysaccharide (LPS) exposure, can induce lasting changes in brain structure, neurochemistry, and function, a process known as “early-life programming.” These changes are associated with neuropsychiatric disorders, including depression. While the nitric oxide (NO) pathway is implicated in depression, how changes in NO kinetics impact sex-dependent affective behaviors has not been thoroughly investigated. This study focuses on the effects of LPS-induced early immune challenge on NO release kinetics in the neonatal rat brain, as well as the link between these effects and long-term sex- and age-related behavioral changes in adolescence. Two cohorts of neonatal rats were used in the study. The first cohort included 80 male and female rats, which were sacrificed at various times after LPS (250 µg/kg) or phosphate buffer saline (PBS) injection on postnatal day 2 (PND2). Brain regions were collected for biochemical analysis. The second cohort consisted of 20 rats, with equal gender distribution, divided into PBS- and LPS-injected groups. At PND60, these rats underwent behavioral tests (Open Field, Elevated Plus Maze, and Forced Swim), followed by biochemical analysis of brain structures. The findings showed a prolonged elevation of NO following the early immune challenge with LPS in males. Furthermore, behavioral assessments conducted during adolescence demonstrated that male rats consistently showed more pronounced anxiety-like and depressive-like behaviors with higher biomarker levels than females. Understanding these sex-specific NO release patterns and how they affect adolescents could be crucial to understanding the neurochemical processes that underlie the development of depression and other mental health disorders.

Keywords | Neonatal, Early-life programming, Sex differences, Wistar rat, Nitric oxide, Central nervous system, Bacterial endotoxin

Received | June 21, 2024; **Accepted** | August 11, 2024; **Published** | August 30, 2024

***Correspondence** | Laila Ibouzine-Dine, Laboratory of Biology and Health, Neurosciences, Neuroimmunology and Behaviour Unit, Faculty of Science Ibn Tofail University, Kenitra, Morocco; **Email:** Laila.ibouzinedine@uit.ac.ma

Citation | Ibouzine-Dine L, Berkiks I, Mallouk H, Lamtai M, Marzouki ZE, Dimaoui A, Mesfioui A, Hessni AE (2024). Impact of early immune challenge by LPS on nitric oxide release dynamics in the neonatal rat brain: Triggering long-lasting sex- and age-related affective behavioral alterations in adolescence. *Adv. Anim. Vet. Sci.* 12(10): 1976-1988.

DOI | <https://dx.doi.org/10.17582/journal.aavs/2024/12.10.1976.1988>

ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331



Copyright: 2024 by the authors. Licensee ResearchersLinks Ltd, England, UK.

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

The neonatal period is a critical timeframe, during which adverse events can significantly impact the development of the central nervous system, thereby affecting behavior later in life (Dinel *et al.*, 2014). Studies on humans and animals have shown evidence of a connection between the immune system and several neuropathological disorders, such as anxiety, depression, and cognitive dysfunctions, which are thought to have a developmental origin (Benmhammed *et al.*, 2019). However, the pathways through which these changes occur are still being explored. Indeed, major depressive-disorder often manifests early during adolescence, a period characterized by significant hormonal fluctuations (Milivojevic and Covault, 2013) and the development of brain regions such as the hippocampus, amygdala, and prefrontal cortex (Patel *et al.*, 2021; Nassiri *et al.*, 2023).

In this context, previous studies in our laboratory have identified critical windows during development when the immune system response to challenges, such as those induced by lipopolysaccharide (LPS), can have significant effects on adult behavior (Berkiks *et al.*, 2018; Benmhammed *et al.*, 2019; Abdeljabbar *et al.*, 2023). These effects may be influenced by various factors, including the timing of the immune challenge, the sex of the individual, and the specific brain regions affected.

Furthermore, the role of inflammatory cytokines, such as interleukin-1 β (IL-1 β), in mediating these behavioral changes has been highlighted; previous studies have shown that neonatal exposure to *E. coli* infection on postnatal day 4, significantly increases inflammatory cytokines, such as IL-1 β and TNF-alpha 24 hours post-infection in the serum (Schwarz and Bilbo, 2011; Nakagawa and Chiba, 2015). Interestingly, previous studies have found that nitric oxide (NO) is involved in the cytotoxic effect of TNF-alpha on endothelial cells, suggesting that cytokine stimulation of the production of NO may be a key mechanism through which immune challenges can influence brain function and ultimately impact behavior (Babri *et al.*, 2014). The study found that nitric oxide synthase (NOS) inhibitors, specifically L-NAME and aminoguanidine, as well as sildenafil, exhibited antidepressant-like effects in mice subjected to LPS-induced depressive-like behavior (Tomaz *et al.*, 2014). The depressive-like behavior induced by LPS is time-dependent, typically manifesting 24 hours after administration. This timing aligns with the peak of inflammatory responses and the subsequent behavioral changes observed in the animal model (Bilbo and Schwarz, 2012). Understanding the neonatal immune system and its impact on the brain is crucial during health and disease processes. As a result, it is essential to investigate different markers of inflammation in the newborn brain.

As known, NO is a significant indicator whose level can be used to evaluate the kinetics of the inflammatory response in damaged tissue (Ziaja *et al.*, 2011) and can have pro- or anti-inflammatory actions, as well as neuroprotective or destructive effects, depending on its age-related expression, acute or chronic expression, and concentration (Garry *et al.*, 2015; Picón-Pagès *et al.*, 2019). This paramagnetic gas is naturally produced in different amounts during postnatal development in an age-dependent manner under normal physiological conditions (Ibouzine-Dine *et al.*, 2024). Our previous investigation revealed fluctuations in the levels of NO in developing brains from postnatal days 1 to 8.

Prior research has demonstrated that NO levels can rise quickly in response to an inflammatory trigger during the second week of postnatal development. Early time points, such as 2 and 6 hours, are usually chosen to assess the immune system's immediate reaction and the rapid generation of NO. Later time points, such as 24 and 48 hours, are used to examine how NO levels change as the inflammatory response progresses (Ziaja *et al.*, 2011). The impact of variations in NO kinetics on sex-dependent affective behaviors has not been thoroughly investigated. We propose that the kinetics of the NO response following a neonatal immune challenge differ between males and females and that these different NO levels will be associated with the development of sex-specific emotional behaviors, such as anxiety and depression, during adolescence. This study aimed to map the temporal progression of the immune response by measuring NO levels at various intervals (e.g., 2, 24, 48, and 72 hours post-infection) and to investigate the link between these changes and long-term sex- and age-related behavioral changes in adolescence.

MATERIALS AND METHODS

ANIMALS

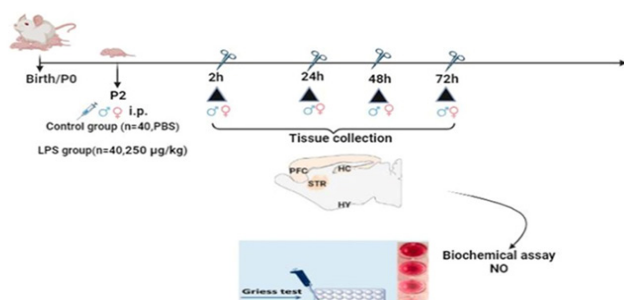
Adult female Wistar rats, weighing 250–280 g, were obtained from the Laboratory of Biology and Health at Ibn Tofail University, acclimatized for one week in the presence of male rats for mating purposes, and then individually housed with unlimited access to food and water under controlled environmental conditions (12-hour light-dark cycle from 7 PM to 7 AM and a temperature of $22 \pm 2^\circ\text{C}$); the newborn rat pups were checked daily at 9:30 AM, with the day of birth designated as postnatal day 0 (PND0) for each pup.

All experimental protocols followed the ethical guidelines published by the National Institutes of Health for the ethical use of laboratory animals in research. The animal ethics committee at Ibn Tofail University (ITU) authorized the protocols used in these experiments. Biochemical studies were carried out at ITU's Biology and Health Laboratory.

LIPOLYSACCHARIDE INJECTIONS AND TISSUE DISSECTIONS

Male and female Wistar rats were utilized in our investigation. On PND2, litters were normalized to 10 pups per litter with an equal distribution of sexes (5 males and 5 females). Subsequently, they received a single intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) (from *Escherichia coli* serotype 026:B6; L-3755, Sigma Aldrich, St. Louis, MO; 250 µg/kg) (Berkiks *et al.*, 2018; Abdeljabbar *et al.*, 2023), or an equivalent volume of PBS for control animals.

Cohort 1



Cohort 2

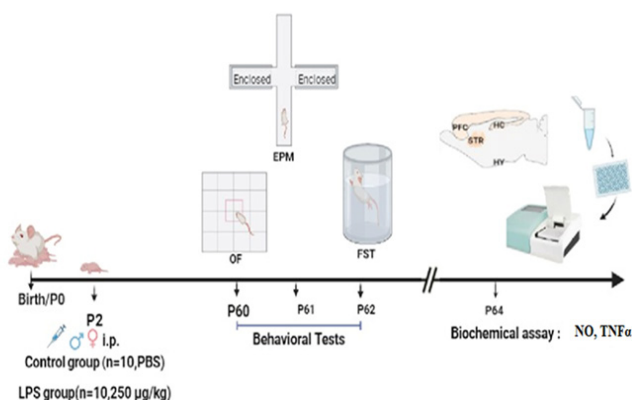


Figure 1: Schematic of the study design and timeline for the experimental process. Two cohorts of rats were employed. In Cohort 1, male (♂ blue) and female (♀ red) Wistar rat pups were sacrificed at 2, 24, 48, and 72 hours post-injection of either (lipopolysaccharide) LPS or PBS (Phosphate Buffer Saline) at postnatal day 2 (P2). The prefrontal cortex (PFC), striatum (STR), hippocampus (HC), and hypothalamus (HY) were dissected to assess nitric oxide (NO) levels. In the second cohort, male and female rats were administered LPS at P2 and subjected to three different behavioral tasks in the following order: open field (OF), elevated plus maze (EPM), and forced swim test (FST) at P60. After sacrifice, brain structures (prefrontal cortex, striatum, hippocampus, and hypothalamus) were collected to assess NO and TNFα levels.

Two cohorts of rats were employed (Figure 1). Cohort 1 was established to investigate the impact of LPS administration on NO production across various brain regions. On postnatal day 2 (P2), a total of 80 male and female rats were

sacrificed at 2 (males n = 5; females n = 5), 24 (males n = 5; females n = 5), 48 (males n = 5; females n = 5), and 72 hours (males n = 5; females n = 5) post-injection of either LPS or PBS. The PBS-treated animals were included as a control group to account for any potential non-specific effects of the injection procedure on NO production. The prefrontal cortex, striatum, hippocampus, and hypothalamus were dissected and homogenized using a Dounce homogenizer in an ice-cold RIPA lysate solution containing 1 mM PMSF. Subsequently, the homogenates underwent centrifugation at 14,000 g for 15 minutes before NO level quantification.

In the second cohort, 20 rats were utilized to explore the impact of neonatal LPS administration on emotional behavior during adolescence (PND60). To accomplish this, rat pups treated with LPS and their respective controls at PND2 underwent three behavioral tests (open field, elevated plus maze, and forced swimming) at PND60. After sacrifice, brain structures (prefrontal cortex, striatum, hippocampus, and hypothalamus) were collected for biochemical analysis.

BEHAVIORAL TESTS

To assess the influence of neonatal infection on affective behavior, we employed the Open Field (OF), Elevated plus Maze (EPM), and Forced Swimming Test (FST). These tests were chosen to provide a comprehensive evaluation of the behavioral consequences of the early-life immune challenge. All behavioral responses were captured using a camera for analysis.

OPEN FIELD TEST

The OF test was used to measure general locomotor activity and exploratory behavior, which can be indicative of underlying anxiety levels. An open square field, measuring 1.2 meters on each side and enclosed by a 40-centimeter-high white wall, was utilized to assess behaviors indicative of anxiety and locomotor activity (Carola *et al.*, 2002).

The field was divided into 25 squares, consisting of 9 central and 16 peripheral squares, each measuring 20 by 20 centimeters. Animals were gently placed in the center of the field and allowed to explore freely for 10 minutes. After each session, the field was cleaned using 70% ethyl alcohol. The parameters assessed included the total distance traveled (reflecting locomotor activity), time spent in the central area, and the frequency of returns to the center (indicative of anxiety levels).

ELEVATED PLUS MAZE TEST

The EPM was chosen to specifically assess anxiety-like behavior by evaluating the natural aversion of rodents to open, elevated spaces. An EPM test was conducted to evaluate anxiety-like behaviors. This test consists of a plus-shaped wooden maze with two open arms (50×10 cm) and two enclosed arms of the same size. The enclosed arms have

wooden walls 40 cm high, except at the center where the arms intersect. The entire maze is elevated 50 cm above the floor. During the test, each animal is placed at the center of the maze, facing one of the enclosed arms, and allowed to explore the maze freely for 5 minutes.

Anxiety-related measures include the time spent by a rat in the open arms and the duration of stretching in the closed arms. Between tests, the apparatus is cleaned using 70% ethyl alcohol (Garcia *et al.*, 2005; Rhaimi *et al.*, 2023).

FORCED SWIM TEST

Depressive mood symptoms were assessed using an FST. Swimming sessions were conducted by placing rats 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 were forced to swim in the cylinder for 5 minutes, and their duration of immobility was recorded. A rat was considered immobile when it ceased all active behaviors (e.g., struggling, swimming, and jumping) and remained passively floating, making minimal movements to maintain its nostrils above water. The latency to the first bout of immobility was also recorded. Increased immobility time is interpreted as an increased depressive-like response (a measure of depression) (Porsolt *et al.*, 1978; Réus *et al.*, 2011).

MEASUREMENT OF BIOCHEMICAL PARAMETERS

After behavioral tests, animals were anesthetized with chloral (100 mg/kg). The four areas were rapidly dissected and homogenized using a Dounce homogenizer in a lysis buffer kept at an ice-cold temperature (RIPA lysate solution + 1 mM PMSF). The homogenates were centrifuged for 15 min at 14000 g, and then TNF α and NO levels were measured.

NITRITE/NITRATE ASSAY

NO conversion to nitrite and nitrate in biological systems enhances nitrite generation. Nitrite represents the stable end product remaining after the auto-oxidation of NO. Thus, evaluating its levels in serum and tissue homogenates is widely recognized as a dependable indicator of NO activity (Naïla *et al.*, 2021; Zghari *et al.*, 2023; Brikat *et al.*, 2024; Harifi *et al.*, 2024). In this investigation, we quantified nitrite concentration utilizing the diazotization method, which relies on the Griess reaction, in rat brain tissue homogenates extracted from the prefrontal cortex, hippocampus, striatum, and hypothalamus. This indirect assay serves as a means to gauge NO production (Chao *et al.*, 1992; El-Hamzaoui *et al.*, 2024).

Tissue samples (500 μl) were dispensed 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 added to each tube. Following a 30-minute incubation at room tempera-

ture, absorbance was recorded at 540 nm. The nitrite concentrations in the tissue homogenates were determined via linear regression analysis, utilizing standard calibration curves generated with sodium nitrite. Tissue nitrite levels were expressed in $\mu\text{mol/g}$ tissue.

TNF α ASSAY

The tumor necrosis factor-alpha (TNF α) content in the homogenates of the prefrontal cortex, hippocampus, striatum, and hypothalamus was measured using an appropriate rat TNF α enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen KRC3011). The TNF α levels were subsequently expressed in pg/ml.

STATISTICAL ANALYSIS

All data were analyzed by two-way ANOVA, followed by Bonferroni's post hoc test for comparison between groups. The data were analyzed using two-way analysis of variance (ANOVA) to assess the main effects of sex (male vs. female) and treatment (LPS vs. PBS), as well as any potential interaction effects between these two factors. This two-way ANOVA approach was selected because the experimental design involved two independent variables (sex and treatment) that could potentially influence the dependent variable NO levels. The data are represented as mean $\pm$ standard error of the mean (SEM) and illustrated by figures produced by the Graph Pad Prism 8 software (Graph Pad Software Inc., La Jolla, California, United States). Significant differences were considered at $p < 0.05$.

RESULTS AND DISCUSSION

KINETICS OF NO PRODUCTION IN RESPONSE TO SYSTEMIC IMMUNE CHALLENGE WITH LPS

PRE-FRONTAL CORTEX: In male rats, LPS injection led to a robust and statistically significant increase in NO levels compared to the PBS control group ($F_{(1, 32)} = 167.2$, $p < 0.0001$), peaking at 2 hours ($F_{(3, 32)} = 32.90$, $p < 0.0001$). During the next 48 hours, the levels of NO were sustained for up to 48 hours before returning to baseline by 72 hours compared to the control (Figure 2).

In female rats, LPS injection also resulted in a significant increase in NO levels compared to the PBS control ($F_{(1, 32)} = 142$, $p < 0.0001$). The peak in NO levels in female LPS-treated rats was observed at 2 hours, similar to the males ($F_{(3, 32)} = 29.29$, $p < 0.0001$). The elevated NO levels in females returned to baseline by 48 hours, earlier than in their male counterparts.

The sex-dependent differences in NO response emerge later, with males showing significantly higher NO levels compared to females at the 48-hour time point, rather than the initial 2-hour period.

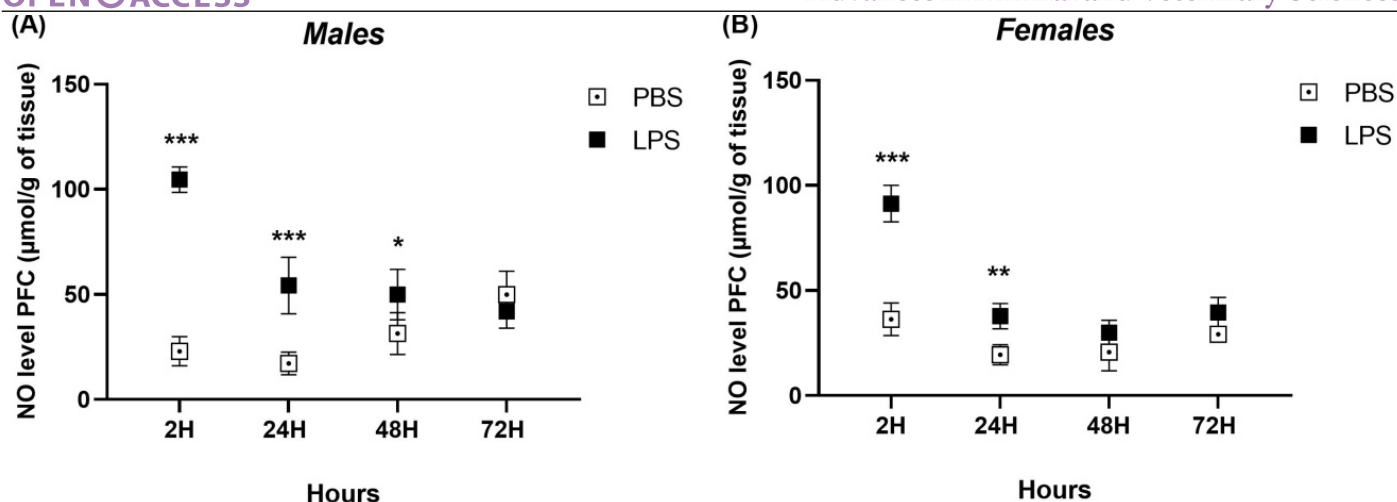


Figure 2: Kinetics of nitric oxide (NO) levels in the prefrontal cortex (PFC) of male (A) and female (B) rats following a single intraperitoneal injection of either lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) as a control over 72 hours in PND2. The significance level is 0.05. * $p < 0.05$ vs. PBS group, according to two-way ANOVA following post-hoc (comparisons Bonferroni's multiple comparisons test).

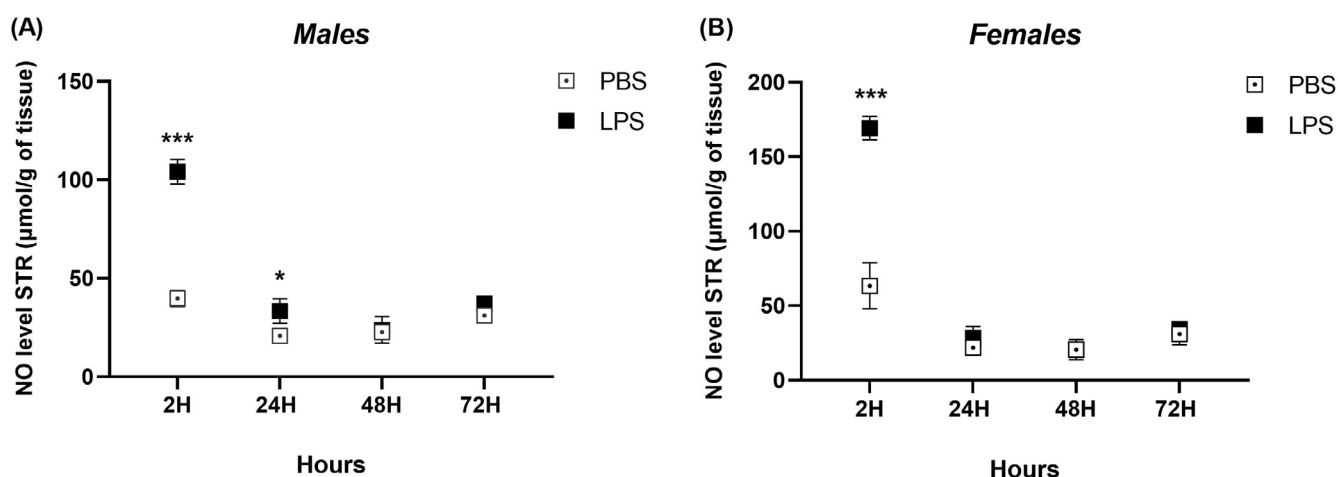


Figure 3: Kinetics of nitric oxide (NO) levels in the striatum (STR) of male (A) and female (B) rats following a single intraperitoneal injection of either lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) as a control over 72 hours in PND2. The significance level is 0.05. * $p < 0.05$ vs. PBS group, according to two-way ANOVA following post-hoc (comparisons Bonferroni's multiple comparisons test).

STRIATUM: In male rats, LPS injection led to a significant increase in NO levels compared to the PBS groups ($F_{(1,32)} = 257.8$, $p < 0.0001$), with the peak response occurring at the 2-hour time point ($F_{(3,32)} = 63.88$, $p < 0.0001$). However, the female rats exhibited a more sustained elevation of NO levels compared to the control group ($F_{(1,32)} = 220.1$, $p < 0.0001$) at 2 and 24-hour time points ($F_{(3,32)} = 67.23$, $p < 0.0001$). After 24 hours, the NO level gradually decline returning to baseline levels by the 48 and 72 hours. In contrast, the PBS group maintains relatively stable and low NO levels throughout the 72-hour time course. Also, data shows a significant difference in striatal NO levels at the 2-hour time point, with females exhibiting higher NO concentrations than males at this early time point (Figure 3).

HIPPOCAMPUS: In male neonatal rats, the LPS group shows a significant increase in hippocampal NO levels compared

to the PBS control ($F_{(1,32)} = 312.5$, $p < 0.0001$) at all-time points (2h,24h,48h,72h). The peak NO response is observed at the 2-hour time point ($F_{(3,32)} = 39.33$, $p < 0.0001$), after which the levels gradually decline but remain elevated compared to controls throughout the 72 hours (Figure 4).

In the female rats, the LPS group also exhibits a significant increase in hippocampal NO levels compared to the PBS ($F_{(1,32)} = 59.49$, $p < 0.0001$), reaching a peak at the 2-hour time point, similar to the males ($F_{(3,32)} = 13.62$, $p < 0.0001$). However, the temporal dynamics differ from the males. The elevated NO levels in the female LPS group return to baseline by the 48-hour time point, whereas the male LPS group maintains higher NO levels throughout the 72-hour time course. The sex-dependent differences in NO show an increase in NO levels in the male LPS group compared to the female LPS group at a 2-hour time point. However, at

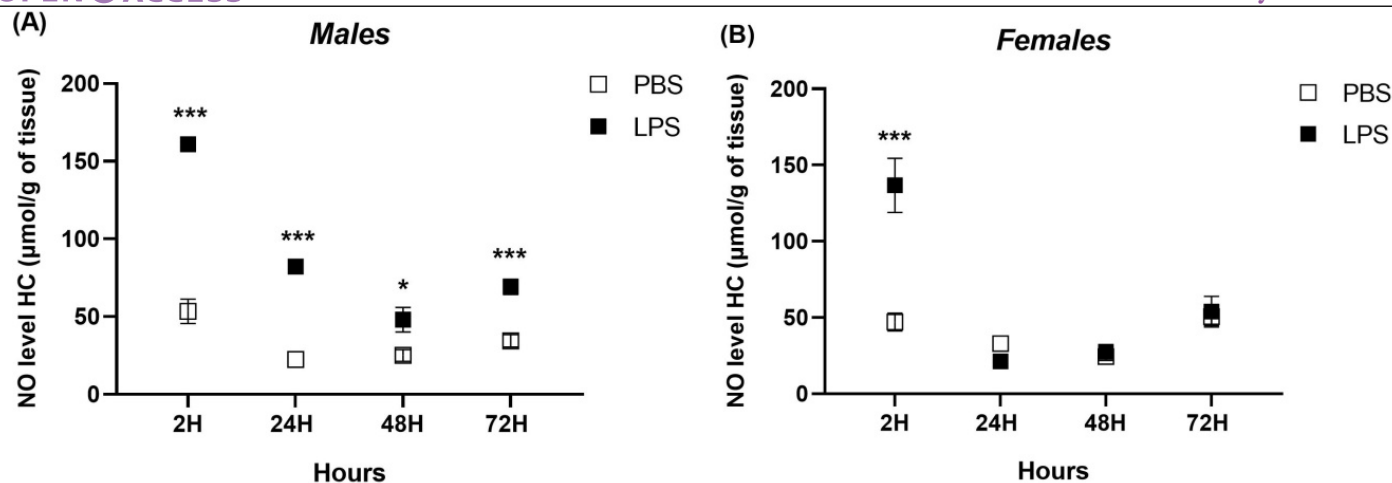


Figure 4: Kinetics of nitric oxide (NO) levels in the hippocampus (HC) of male (A) and female (B) rats following a single intraperitoneal injection of either lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) as a control over 72 hours in PND2. The significance level is 0.05. * $p < 0.05$ vs. PBS group, according to two-way ANOVA following post-hoc (comparisons Bonferroni's multiple comparisons test).

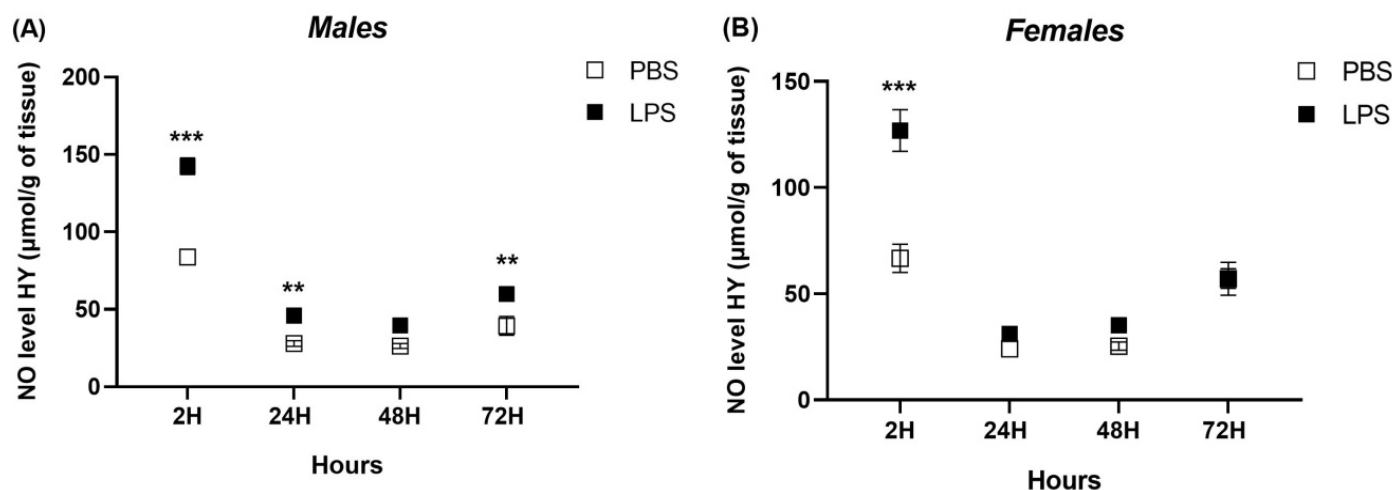


Figure 5: Kinetics of nitric oxide (NO) levels in the hypothalamus (HY) of male and female rats following a single intraperitoneal injection of either lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) as a control over 72 hours in PND2. The significance level is 0.05. * $p < 0.05$ vs. PBS group, according to a two-way ANOVA following post-hoc (comparisons Bonferroni's multiple comparisons test).

the 24, 48, and 72-hour time points; there is no significant difference in hippocampal NO levels between the male and female LPS groups.

HYPOTHALAMUS: In the male rats, the LPS group shows a significant increase ($F_{(1,32)} = 293, p < 0.0001$) in hypothalamic NO levels compared to the PBS control at all time points (2h, 24h, 48h, and 72h). The peak NO response is observed at the 2-hour time point in the male LPS group ($F_{(3,32)} = 9.102, p = 0.0002$), after which the levels gradually decline but remain elevated compared to controls throughout the 72 hours. In the female rats, the LPS group also exhibits a significant increase ($F_{(1,32)} = 64.00, p < 0.0001$) in hypothalamic NO levels compared to the PBS control, but the temporal dynamics differ from the males. The female LPS group reaches a peak in NO levels at the 2-hour time point ($F_{(3,32)} = 20.27, p < 0.0001$), similar to the males.

However, the increased NO levels in the female LPS group return to baseline by the 48-hour time point, whereas the male LPS group maintains higher NO levels throughout the 72-hour time course. Also, data shows a significant difference ($F_{(1,32)} = 39.55, p < 0.0001$) in hypothalamic NO levels between the male and female LPS groups at 24 and 48-hour time points. However, at the 48-hour time point, the male LPS group shows significantly higher NO levels compared to the female LPS group (Figure 5).

IMPACT OF EARLY IMMUNE CHALLENGE ON THE ANXIETY-LIKE BEHAVIOR EVALUATED IN THE OFT

Our findings indicate that male rats treated with LPS exhibited significantly higher levels of anxiety-like behaviors compared to the control rats ($F_{(1,62)} = 27.31; p < 0.0001$). Specifically, the LPS-treated male rats spent less time in the central area of the open field, made fewer entries into

the center, and displayed lower overall locomotor activity - all of which suggest elevated anxiety levels. In contrast, the female rats show less significant differences in these anxiety-related parameters between the LPS-treated and control rats. These results highlight the sex-dependent nature of the effects induced by LPS treatment, with the male adolescent rats demonstrating a more pronounced anxiety-like phenotype in the open field test compared to their female counterparts (Figure 6).

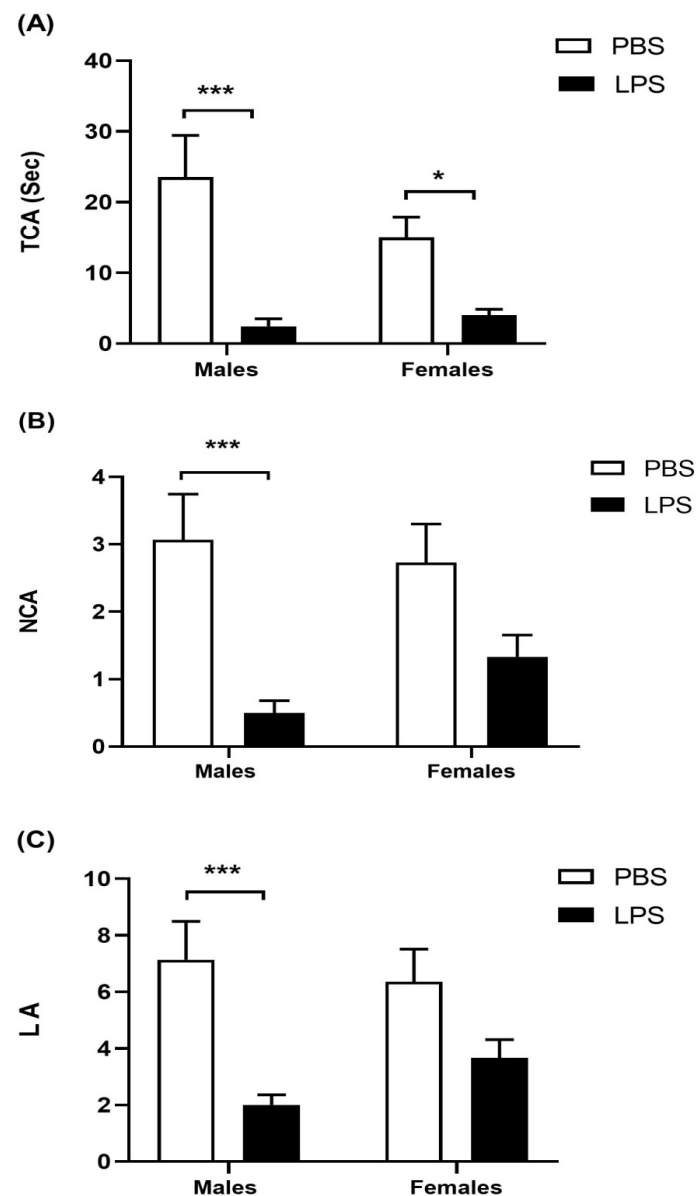


Figure 6: Behavioral performances of male and female rats in the open field test. A) Total amount of time spent in the central area (TCA) measured in seconds, B) Number of entries into the center area (NCA), C) Locomotor Activity (LA). The data are presented as means; $\pm$ SEM. * $p < 0.05$ vs. PBS group; and # $p < 0.05$ vs. gender counterpart.

IMPACT OF EARLY IMMUNE CHALLENGE ON THE ANXIETY-LIKE BEHAVIOR EVALUATED IN THE EPM

The results from the elevated plus maze test demonstrate that the male adolescent rats displayed more pronounced

anxiety-like behaviors compared to the female rats. Specifically, the male rats spent significantly less time in the open arms compared to the control rats, made fewer entries into the open areas, and exhibited lower overall locomotor activity levels within the test environment ($F_{(1, 68)} = 5,592$, $p = 0,0209$). These findings parallel the previous observations from the open field test, further highlighting the sex-dependent nature of the anxiety-related responses in this rodent model. Collectively, the data suggests the male adolescent rats exhibited heightened levels of anxiety-like phenotypes across multiple behavioral measures, in contrast to their female counterparts (Figure 7).

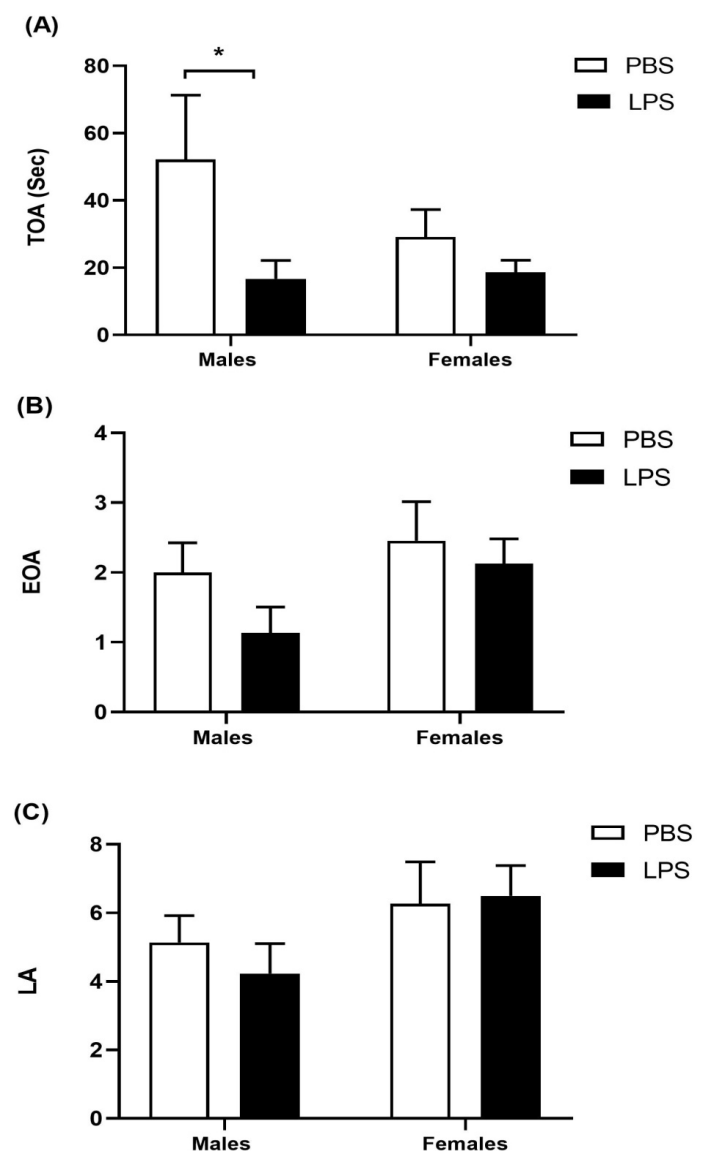


Figure 7: Behavioral performance of male and female rats measured in the elevated plus maze test. A) Total amount of time spent in the open arms (TOA), measured in seconds; B) Number of entries in the open arms (EOA); C) Locomotor Activity (LA). The data are presented as means $\pm$ SEM. * $p < 0.05$ vs. PBS group; and # $p < 0.05$ vs. gender counterpart.

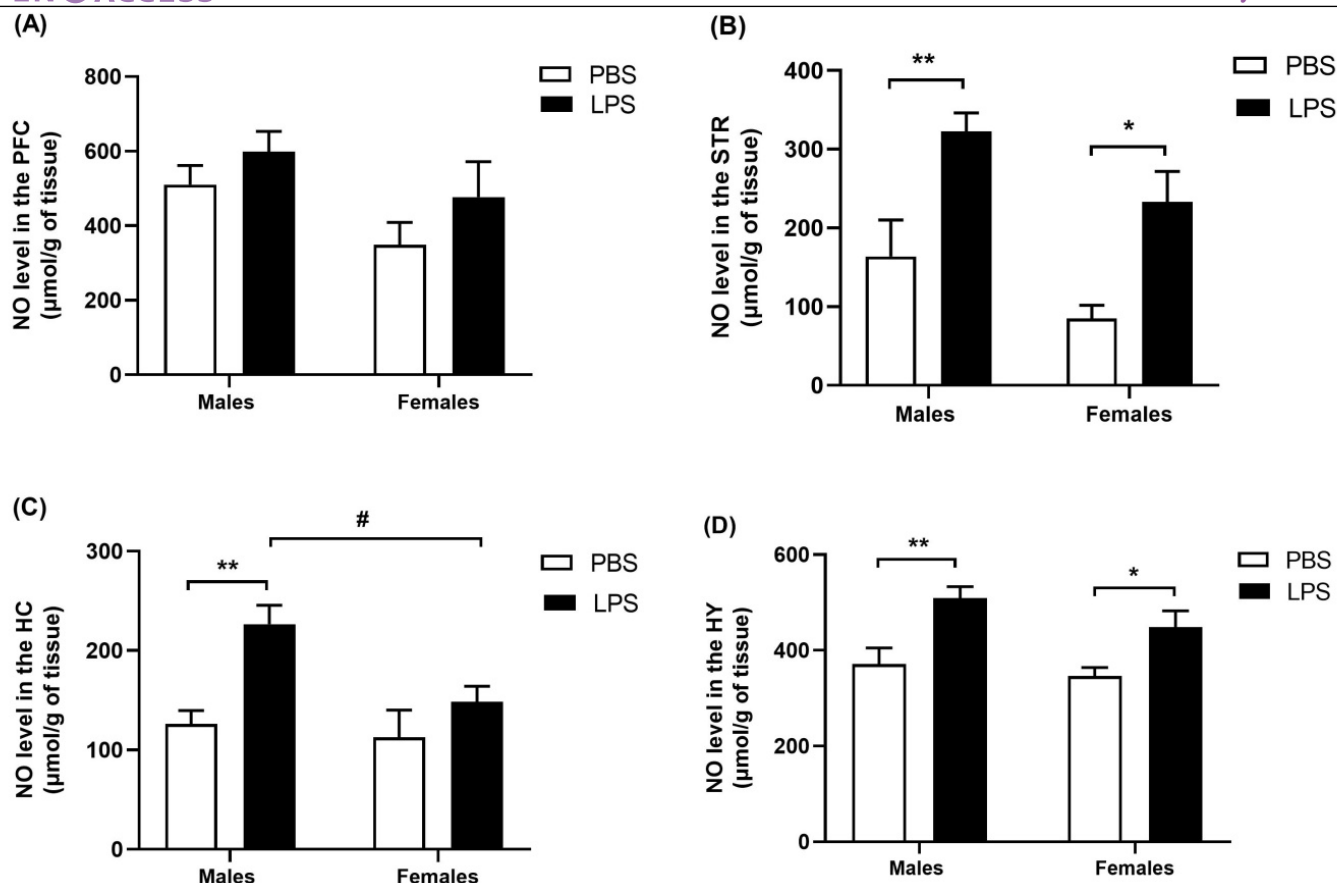


Figure 9: Concentrations of nitric oxide (NO) in different regions of male and female rat brain. A) NO level in the prefrontal cortex (PFC). B) NO level in the hippocampus (HC). C) NO level in the striatum (STR). D) NO level in the hypothalamus (HY). The data are presented as means $\pm$ SEM. * $p < 0.05$ vs. PBS group; and # $p < 0.05$ vs. gender counterpart.

IMPACT OF EARLY IMMUNE CHALLENGE ON DEPRESSIVE-LIKE BEHAVIOR EVALUATED IN THE FST

Our results showed that the male rats exhibited higher levels of immobility time in the forced swimming test compared to control rats, suggesting a more pronounced behavioral despair or depressive-like phenotype ($F_{(1, 67)} = 8,052$, $p = 0,006$). In contrast, the female rats showed a smaller increase in their total time of immobility compared to the control, indicating a sex-dependent effect of the LPS treatment on this particular behavioral measure (Figure 8).

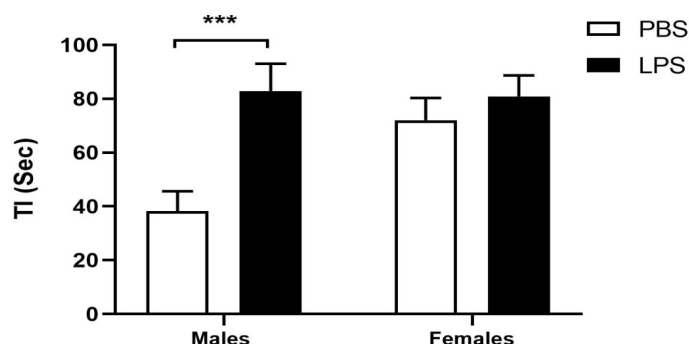


Figure 8: Behavioral performance of male and female rats measured in the forced swimming test. Total time of immobility (TI), measured in seconds. The data are presented as means $\pm$ SEM. * $p < 0.05$ vs. PBS group; and # $p < 0.05$ vs. gender counterpart.

IMPACT OF EARLY IMMUNE CHALLENGE ON NO AND TNF α LEVELS IN DIFFERENT REGIONS OF THE RAT BRAIN

EFFECT OF NO LEVELS: In the prefrontal cortex, both the male and female rats showed an increase in NO levels following LPS treatment compared to their respective control. However, the increase was more pronounced in the male rats compared to the female rats (Figure 9A). Similarly, in the hippocampus, the male rats showed a significant increase in NO concentrations ($F_{(1, 16)} = 5,483$, $p = 0,0325$) with LPS treatment, while the female rats showed a relatively smaller increase (Figure 9C). The same pattern was observed in the striatum (Figure 9B) and hypothalamus (Figure 9D), where the male rats displayed a robust increase in NO concentrations with LPS treatment, while the female rats showed a relatively smaller increase.

EFFECT OF TNF α LEVELS: The results demonstrate a clear sex-dependent difference in the neuroinflammatory response to LPS treatment in adolescent rats. In the prefrontal cortex (Figure 10A), the male rats showed a significantly higher TNF α level in the LPS-treated group compared to the control ($F_{(1, 16)} = 12,18$, $p = 0,0030$). In contrast, the female rats did not exhibit a significant difference in PFC TNF α levels between the LPS and PBS groups. A similar pattern was observed in the hippocampus

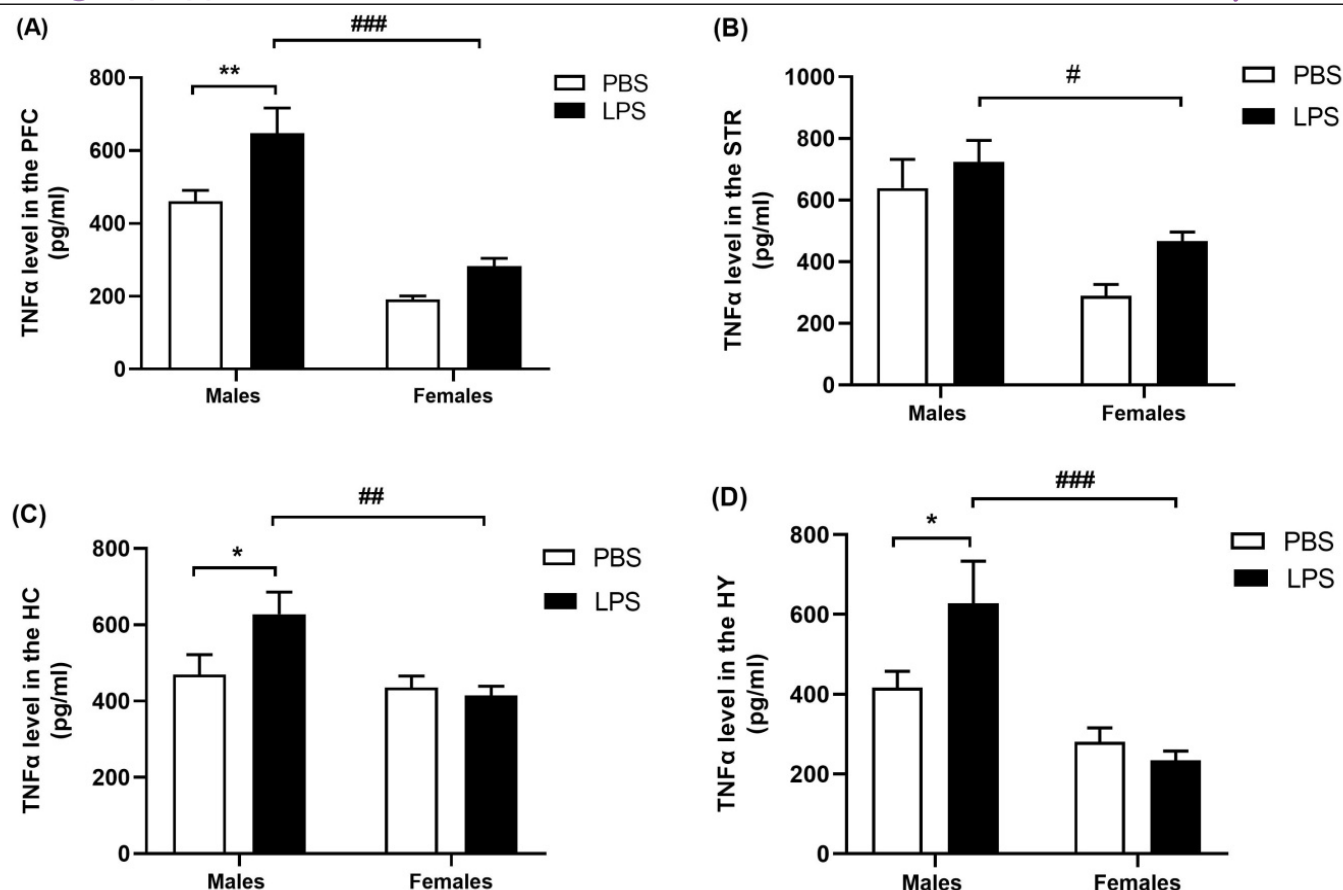


Figure 10: Concentrations of TNF alpha (TNF α) in different regions of male and female rat brain. A) NO level in the prefrontal cortex (PFC). B) NO level in the hippocampus (HC). C) NO level in the striatum (STR). D) NO level in the hypothalamus (HY). The data are presented as means $\pm$ SEM. * $p < 0.05$ vs. PBS group; and # $p < 0.05$ vs. gender counterpart.

(Figure 10C), where the male LPS-treated rats displayed a more pronounced increase in TNF α concentrations compared to male control rats. The same trend continued in the striatum (Figure 10B) and hypothalamus (Figure 10D), with the male LPS-treated rats demonstrating a greater elevation in TNF α levels compared to their controls. Also, the data reveals a sex-dependent difference in the neuroinflammatory response, where the adolescent male rats showed a markedly greater elevation in the levels of the pro-inflammatory mediator TNF α compared to the adolescent female rats across the examined brain regions.

These findings extend previous studies that have reported sex-specific differences in neuroinflammatory responses to immune challenges. While earlier work has focused on cytokine profiles, our results reveal that the temporal and regional patterns of NO production also exhibit robust sex differences, which may have important implications for understanding neuropsychiatric disorders with known sex biases.

The present studies highlight the significant impact of early immune challenge on NO production in different regions of the rat brain. Additionally, we investigated the correlation between these neurochemical changes and behavior

alterations observed during adolescence. The results of the first study reveal some key aspects of NO production in neonatal animals during an early immune challenge by LPS. The findings showed that both male and female rat pups exhibited significant increases in NO levels across all examined brain regions following LPS injection on PND2. However, the timing and magnitude of these NO changes varied between the sexes and different brain areas. The study provides important insights into the sex-specific and region-specific patterns of NO production in the developing brain during an early immune insult.

The results of the first study reveal some key aspects of NO production in neonatal animals during an early immune challenge by LPS. The findings showed that both male and female rat pups exhibited significant increases in NO levels across all examined brain regions following LPS injection on PND2. However, the timing and magnitude of these NO changes varied between the sexes and different brain areas. The study provides important insights into the sex-specific and region-specific patterns of NO production in the developing brain during an early immune insult.

Our previous research showed variations in NO levels in developing brains from postnatal days 1 to 17 (Ibou-

zine-Dine *et al.*, 2024). Previous research has found that the amount of NO generated varies by brain area, with the cortex, hippocampus, hypothalamus, substantia nigra, and amygdala producing the most (Chachlaki *et al.*, 2017). Some studies have shown that substantial regional differences in brain NO levels have been observed in response to the LPS challenge. For example, the degree of NOS inhibition varied markedly between brain regions within each hemisphere and correlated with their ventricular proximity to the site of NOS expression (Salter *et al.*, 1995). Our findings revealed that in the prefrontal cortex, both male and female rats exhibited a robust and significant increase in NO levels following LPS injection but the temporal patterns differed, with males showing a more prolonged elevation of NO that persisted for up to 48 hours, while females returned to baseline by 48 hours. Similar to the prefrontal cortex, both sexes showed an increased NO level after LPS injection in the hypothalamus. Previously, it was demonstrated that the PFC exhibits a heightened neuroinflammatory response to LPS challenge in the context of stress, as evidenced by the increased production of proinflammatory cytokines (Cerqueira *et al.*, 2007), other study demonstrated that the hypothalamus also plays a key role in the neuroimmune response to LPS, as evidenced by the induction of inflammatory gene expression such as iNOS (Cazareth *et al.*, 2014; Adzic *et al.*, 2015). In the striatum, Females displayed a more prolonged increase in NO levels, remaining elevated at both 2 and 24 hours after LPS injection. In contrast, males showed a peak increase at 2 hours, returning to baseline by 48 hours. Our results suggest that NO may serve as an important mediator of sex differences in the neuroinflammatory response to immune challenges in the brain. The striatum is known to express high levels of neuronal NO synthase (nNOS); however, previous studies have shown that dopamine (DA)-glutamate interactions in the striatum can stimulate nNOS activity, leading to increased NO production (Park and West, 2009). In female rats, the augmented and prolonged NO response in the striatum following LPS exposure may contribute to sex differences in the regulation of motor function and the susceptibility to neuropsychiatric disorders associated with striatal dysfunction (Reis *et al.*, 2017). Additionally, we found that in the striatum, males exhibited a significant increase in NO that was sustained for up to 48 hours after LPS treatment, whereas females showed a more transient elevation that returned to baseline by 24 hours (Jin *et al.*, 2017; Reis *et al.*, 2017). Also, our results demonstrated sex-dependent differences in NO levels in response to LPS treatment in the hippocampus. Specifically, in the 2-hour time point post-injection, the male LPS group exhibited a more pronounced increase in NO levels compared to the female LPS group. Our findings align with a recent study that examined sex differences in the temporal dynamics of TNF α levels following an immune challenge. The researchers found that while both male and female mice exhibited

peak TNF α concentrations at 6 hours post-challenge, this heightened response persisted for at least 24 hours only in the male mice. In contrast, female mice showed a more rapid elevation and faster resolution of cytokine activity compared to their male counterparts (Finnell *et al.*, 2023). These results suggest that sex influences not only the magnitude but also the kinetics of the neuroimmune response, with males demonstrating a more prolonged pro-inflammatory state following immune activation.

Importantly, there were also significant sex differences in inflammatory mediators at baseline (without LPS challenge) in the neonatal animals (Hedley *et al.*, 2023; Ibou-zine-Dine *et al.*, 2024). The present study's discrepancies could be related to the PND period by which LPS was administered, beyond dose. For example Berkiks *et al.* (2018) demonstrated that administering LPS to rats at a dose of 250 μ g/kg on PND 14 increased cytokine levels and ROS after 48 hours. The results of the second study further reinforce the hypothesis that the sex-specific differences in the neurochemical response, particularly the prolonged elevation of NO following the early immune challenge with LPS, serve as a critical underlying mechanism driving the subsequent emergence of long-lasting, sex-dependent anxiety-like and depressive-like behaviors in adolescent rats. Male rats consistently showed more pronounced anxiety-like behaviors and higher immobility times in the FST, while female rats demonstrated less variability in these parameters. This suggests that the sex-dependent differences in the long-term anxiety-like and behavioral despair-related outcomes observed in this model may be attributable to the prolonged elevation of NO levels, specifically in the male rats, during the neonatal period following the early life immune challenge. These findings correlate with Babri *et al.* (2014) which found that postnatal exposure to high doses of the inflammatory cytokine TNF- α increased anxiety- and depression-related behaviors in adult male mice but decreased anxiety without affecting depression in adult female mice. Intriguingly, the sex-specific impact on emotional behaviors observed in the current study, as well as the previous research using a different inflammatory challenge, suggests that the adolescent immune challenge may have the capacity to perturb the normative development of neural circuits underlying emotional regulation in a sex-dependent manner (Granata *et al.*, 2022).

Additionally, our results of neurochemical analysis revealed increased NO levels in both male and female rats across the examined brain regions. However, the response is notably sex-dependent, with male rats consistently exhibiting more pronounced increases in NO concentrations compared to their female counterparts. This suggests that male rats may have a heightened neurochemical response to early immune challenge. Importantly, the data also reveals a sex-depend-

ent difference in the neuroinflammatory response, where the adolescent male rats showed a markedly greater elevation in the levels of the pro-inflammatory mediator TNF α compared to the adolescent female rats in the prefrontal cortex, hippocampus, striatum, and hypothalamus. The elevated TNF α levels in the male rats may contribute to the more pronounced increases in NO observed in this group. TNF α is known to stimulate the production of NO, a key inflammatory signaling molecule in the brain (Koenig *et al.*, 2018; Nonoguchi *et al.*, 2022). Therefore, the sex-dependent differences in the neuroinflammatory response, as evidenced by the divergent patterns of TNF α and NO, suggest that the male adolescent rats mounted a more robust neurochemical reaction to the LPS challenge. Previous research has indicated that oxidative and nitrosative stresses (O and NS) play a significant role in the development of anxiety-like disorders. In this context, it has been found that anxiolytic medications reduce NO levels in animals (Arabi *et al.*, 2021). Extensive evidence indicates that the activation of the neuro-immune response and neuroinflammation is crucial in the pathophysiology of mood disorders, including anxiety (Abelaira *et al.*, 2021).

Previous research has demonstrated sex differences in the neuroimmune response, with males typically exhibiting a more robust neuroinflammatory response to immune challenges compared to females (MacRae *et al.*, 2015; Nelson and Lenz, 2017). A study by Hedley *et al.* (2023) showed a distinct sex-specific response in the expression of all measured inflammatory mediators in the brainstem, at both the neonatal (day 7) and adolescent (day 60) time points. Also, some studies suggest that the hypothalamus is particularly sensitive to changes in NO signaling during inflammation, exhibiting higher NOS activity compared to other brain regions (Burfeind *et al.*, 2016). Inducible NOS (iNOS) is robustly activated in hypothalamic macrophages during chronic inflammation, leading to the production of NO and contributing to hypothalamic inflammation (Lee *et al.*, 2018).

CONCLUSIONS AND RECOMMENDATIONS

In summary, this biochemical investigation reveals more precise information on NO kinetics in the prefrontal cortex, the striatum, the hippocampus, and the hypothalamus of female and male rats. It also revealed the timing of critical changes in NO levels during postnatal development. These findings provide important insights into the sex-specific and region-specific patterns of NO production in the developing brain during an early immune insult, and how these neurochemical changes may contribute to the subsequent emergence of sex-dependent behavioral alterations.

I would like to express my sincere gratitude to the Neurosciences, Neuroimmunology, and Behavioral Unit for their invaluable assistance in the preparation of this article. Their expertise and support have greatly contributed to the development of this research.

NOVELTY STATEMENT

This study uniquely demonstrates that early immune challenges, specifically LPS exposure during the neonatal period, lead to sex-specific alterations in NO production that are critical for the development of long-lasting anxiety-like and depressive-like behaviors in adolescent rats. By elucidating the differential kinetics of NO levels in male and female brains and correlating these changes with distinct behavioral outcomes, this research provides new insights into the neurobiological mechanisms underlying sex differences in the neuroimmune response and their implications for understanding the etiology of affective disorders.

ABBREVIATIONS

CNS= Central Nervous System
ELISA= Enzyme-Linked Immunosorbent Assay
iNOS= Inducible Nitric Oxide Synthase
NADPH-d= Nicotinamide Adenine Dinucleotide Phosphate-diaphorase
NO= Nitric Oxide
nNOS= Neuronal Nitric Oxide Synthase
O and NS= oxidative and nitrosative stresses
PMSF= Phenylmethylsulfonyl fluoride
PND= Postnatal Day
ROS= Reactive Oxygen Species
TNF α = Tumor Necrosis Factor Alpha

AUTHOR'S CONTRIBUTIONS

All authors contributed equally to the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Abdeljabbar N, Miloud C, Inssaf B, Hajar B, Mouloud L, Latifa C, Abdelhalem M, Aboubaker EH (2023). Sex Dimorphism of Memory Response to Long-term Effect Lipopolysaccharide Administration in Wistar Rats. *Int. J. Chem. Biochem. Sci.*, 24:685–6982.
- Abelaira HM, Veron DC, de Moura AB, Carlessi AS, Borba LA, Botelho MEM, Andrade NM, Martinello NS, Zabet GC, Joaquim L, Biehl E, Bonfante S, Budni J, Petronilho F, Quevedo J, Réus GZ (2021). Sex differences on the behavior and oxidative stress after ketamine treatment in adult rats subjected to early life stress. *Brain Res. Bull.*, 172:129–138.

- <https://doi.org/10.1016/j.brainresbull.2021.04.021>
- Adzic M, Djordjevic J, Mitic M, Brkic Z, Lukic I, Radojicic M (2015). The contribution of hypothalamic neuroendocrine, neuroplastic and neuroinflammatory processes to lipopolysaccharide-induced depressive-like behaviour in female and male rats: Involvement of glucocorticoid receptor and C/EBP- β . *Behav. Brain Res.*, 291:130–139. <https://doi.org/10.1016/j.BBR.2015.05.029>
- Arabi M, Nasab SH, Lorigooini Z, Boroujeni SN, Mortazavi SM, Anjomshoa M, Amini-Khoei H (2021). Auroptene exerts protective effects on maternal separation stress-induced changes in behavior, hippocampus, heart and serum of mice. *Int. Immunopharmacol.*, 93:107436. <https://doi.org/10.1016/j.intimp.2021.107436>
- Babri S, Doosti MH, Salari AA (2014). Tumor necrosis factor- α during neonatal brain development affects anxiety- and depression-related behaviors in adult male and female mice. *Behav. Brain Res.*, 261:305–314. <https://doi.org/10.1016/j.BBR.2013.12.037>
- Benmhammed H, El Hayek S, Nassiri A, Bousalham R, Mesfioui A, Ouichou A, El Hessni A (2019). 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.*, 362:46–55. <https://doi.org/10.1016/j.bbr.2019.01.005>
- Berkiks I, Benmhammed H, Mesfioui A, Ouichou A, El hasnaoui A, Mouden S, Touil T, Bahbiti Y, Nakache R, El hessni A (2018). Postnatal melatonin treatment protects against affective disorders induced by early-life immune stimulation by reducing the microglia cell activation and oxidative stress. *Int. J. Neurosci.*, 128:495–504. <https://doi.org/10.1080/00207454.2017.1398156>
- Bilbo SD, Schwarz JM (2012). The immune system and developmental programming of brain and behavior. *Front. Neuroendocrinol.*, 33:267–286. <https://doi.org/10.1016/j.YFRNE.2012.08.006>
- Brikat S, Lamtai M, Chakit M, Ibouazine-Dine L, Fitah I, Abouyaala O, Mesfioui A, El Hessni A (2024). Curcuma Longa Methanolic Extract and Losartan Improves Memory Impairment and Oxidative Stress induced by a High Caloric Diet in Wistar Rats. *Adv. Anim. Vet. Sci.*, 12(4):614–623. <https://doi.org/10.17582/journal.aavs/2024/12.4.614.623>
- Burfeind KG, Michaelis KA, Marks DL (2016). The central role of hypothalamic inflammation in the acute illness response and cachexia. *Seminars cell dev. Biol.*, 54: 42. <https://doi.org/10.1016/j.SEMCDB.2015.10.038>
- Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P (2002). Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behav. Brain Res.*, 134: 49–57. [https://doi.org/10.1016/S0166-4328\(01\)00452-1](https://doi.org/10.1016/S0166-4328(01)00452-1)
- Cazareth J, Guyon A, Heurteaux C, Chabry J, Petit-Paitel A (2014). Molecular and cellular neuroinflammatory status of mouse brain after systemic lipopolysaccharide challenge: importance of CCR2/CCL2 signaling. *J. Neuroinflammation.*, 11: 132. <https://doi.org/10.1186/1742-2094-11-132>
- Cerqueira JJ, Mailliet F, Almeida OFX, Jay TM, Sousa N (2007). The prefrontal cortex as a key target of the maladaptive response to stress. *J. Neurosci.*, 27: 2781–2787. <https://doi.org/10.1523/JNEUROSCI.4372-06.2007>
- Chachlaki K, Malone SA, Qualls-creekmore E, Münzberg H, Giacobini P, Ango F (2017). Phenotyping of nNOS Neurons in the Postnatal and Adult Female Mouse Hypothalamus. *J. Comp. Neurol.*, 525(15): 3177–3189. <https://doi.org/10.1002/cne>
- Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK (1992). Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J. Immunol.*, 149: 2736–2741. <https://doi.org/10.4049/jimmunol.149.8.2736>
- Dinel A-L, Joffre C, Trifileff P, Aubert A, Foury A, Le Ruyet P, Layé S (2014). Inflammation early in life is a vulnerability factor for emotional behavior at adolescence and for lipopolysaccharide-induced spatial memory and neurogenesis alteration at adulthood. *J. neuroinflammation.*, 11: 155. <https://doi.org/10.1186/s12974-014-0155-x>
- El-Hamzaoui A, Lamtai M, Ibouazine-Dine L, Azirar S, El-Brouzi MY, Rezaoui A, El-Hessni A, Mesfioui A (2024). Dose-Dependent Effects of Subchronic Exposure to Glyphosate-Based Herbicide on Behavior and Biochemical Alterations in Adult Rats. *Adv. Anim. Vet. Sci.*, 12(6): 1047–1054. <https://doi.org/10.17582/journal.aavs/2024/12.6.1047.1054>
- Finnell JE, Speirs IC, Tronson NC (2023). Sex differences in hippocampal cytokine networks after systemic immune challenge. *bioRxiv.*, 378257. <https://doi.org/10.1101/378257>
- Garcia AMB, Cardenas FP, Morato S (2005). Effect of different illumination levels on rat behavior in the elevated plus-maze. *Physiol. Behav.*, 85: 265–270. <https://doi.org/10.1016/j.PHYSBEH.2005.04.007>
- Garry PS, Ezra M, Rowland MJ, Westbrook J, Pattinson KTS (2015). The role of the nitric oxide pathway in brain injury and its treatment - From bench to bedside. *Exp. Neurol.*, 263:235–243. <https://doi.org/10.1016/j.expneurol.2014.10.017>
- Granata L, Gildawie KR, Ismail N, Brenhouse HC, Kopec AM (2022). Immune signaling as a node of interaction between systems that sex-specifically develop during puberty and adolescence. *Dev. Cogn. Neurosci.*, 57: 101143. <https://doi.org/10.1016/j.DCN.2022.101143>
- Harifi H, Lamtai M, Salhi SA, Azzaoui F-Z, Akhouayri O, Mesfioui A, Bikjdaouene L (2024). Toxicity Assessment Due to Chronic Exposure to Manganese in Male Wistar Rat. *Adv. Anim. Vet. Sci.*, 12(8): 1509–1516. <https://doi.org/10.17582/journal.aavs/2024/12.8.1509.1516>
- Hedley KE, Cuskelly A, Callister RJ, Horvat JC, Hodgson DM, Tadros MA (2023). Autonomic regions of the brainstem show a sex-specific inflammatory response to systemic neonatal lipopolysaccharide. *bioRxiv.*, 2023.06.14.544893. <https://doi.org/10.1101/2023.06.14.544893>
- Ibouazine-Dine L, Berkiks I, Lamtai M, Mallouk H, Rezaoui A, Nassiri A, Mesfioui A, El Hessni A (2024). Age and Gender-Dependent in Nitric Oxide Postnatal Change Activity in the Rat Brain. *Adv. Anim. Vet. Sci.*, 12: 915–922. <https://doi.org/10.17582/journal.aavs/2024/12.5.915.922>
- Jin X, Yu ZF, Chen F, Lu GX, Ding XY, Xie LJ, Sun JT (2017). Neuronal nitric oxide synthase in neural stem cells induces neuronal fate commitment via the inhibition of histone deacetylase 2. *Front. Cell. Neurosci.*, 11: 1–12. <https://doi.org/10.3389/fncel.2017.00066>
- Koenig S, Bredehöft J, Perniss A, Fuchs F, Roth J, Rummel C (2018). Age dependent hypothalamic and pituitary responses to novel environment stress or lipopolysaccharide in rats. *Front. Behav. Neurosci.*, 12: 343528. <https://doi.org/10.3389/FNBEH.2018.00055/BIBTEX>
- Lee CH, Kim HJ, Lee YS, Kang GM, Lim HS, Lee S hwan, Song DK, Kwon O, Hwang I, Son M, Byun K, Sung YH, Kim S, Kim JB, Choi EY, Kim YB, Kim K, Kweon MN, Sohn

- JW, Kim MS (2018). Hypothalamic Macrophage Inducible Nitric Oxide Synthase Mediates Obesity-Associated Hypothalamic Inflammation. *Cell reports.*, 25: 934. <https://doi.org/10.1016/j.CELREP.2018.09.070>
- MacRae M, Macrina T, Khoury A, Migliore MM, Kentner AC (2015). Tracing the trajectory of behavioral impairments and oxidative stress in an animal model of neonatal inflammation. *Neuroscience.*, 298: 455–466. <https://doi.org/10.1016/j.NEUROSCIENCE.2015.04.048>
- Milivojevic V, Covault J (2013). Alcohol Exposure During Late Adolescence Increases Drinking in Adult Wistar Rats, an Effect that is not Reduced by Finasteride. *Alcohol.*, 48(1): 28–38. <https://doi.org/10.1093/alc/alg105>
- Naïla N, Makthar W, Lamtai M, Zghari O, Aboubaker EH, Abdelhalem M, Ali O (2021). Effect of intra-hippocampal lead injection on affective and cognitive disorders in male WISTAR rats: Possible involvement of oxidative stress. *E3S Web Conf.*, 319: 02017. <https://doi.org/10.1051/e3sconf/202131902017>
- Nakagawa Y, Chiba K (2015). Diversity and plasticity of microglial cells in psychiatric and neurological disorders. *Pharmacol. Ther.*, 154: 21–35. <https://doi.org/10.1016/j.pharmthera.2015.06.010>
- Nassiri A, Lamtai M, Berkiks I, Benmhammed H, Coulibaly M, Chakit M, Mesfioui A, Hessni A El (2023). Age and Sex-Specific Effects of Maternal Deprivation on Memory and Oxidative Stress in the Hippocampus of Rats. *Int. J. Chem. Biochem. Sci.*, 24: 121–129.
- Nelson LH, Lenz KM (2017). The immune system as a novel regulator of sex differences in brain and behavioral development. *J. Neurosci. Res.*, 95: 447–461. <https://doi.org/10.1002/JNR.23821>
- Nonoguchi HA, Kouo TWS, Kortagere S, Hillman J, Boyle DL, Mandyam CD (2022). Lipopolysaccharide Exposure Differentially Alters Plasma and Brain Inflammatory Markers in Adult Male and Female Rats. *Brain Sci.*, 12: 972. <https://doi.org/10.3390/BRAINSCI12080972>
- Park DJ, West AR (2009). Regulation of striatal nitric oxide synthesis by local dopamine and glutamate interactions. *J. Neurochem.*, 111: 1457–1465. <https://doi.org/10.1111/J.1471-4159.2009.06416.X>
- Patel PK, Leatham LD, Currin DL, Karlsgodt KH (2021). Adolescent Neurodevelopment and Vulnerability to Psychosis. *Biol. Psychiatry.*, 89: 184–193. <https://doi.org/10.1016/j.biopsych.2020.06.028>
- Picón-Pagès P, Garcia-Buendia J, Muñoz FJ (2019). Functions and dysfunctions of nitric oxide in brain. *Biochim. Biophys. Acta Mol. Basis. Dis.*, 1865: 1949–1967. <https://doi.org/10.1016/j.bbdis.2018.11.007>
- Porsolt RD, Anton G, Blavet N, Jalfre M (1978). Behavioural despair in rats: A new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.*, 47: 379–391. [https://doi.org/10.1016/0014-2999\(78\)90118-8](https://doi.org/10.1016/0014-2999(78)90118-8)
- Reis PA, Albuquerque CFG de, Maron-Gutierrez T, Silva AR, Neto HC de CF, Reis PA, Albuquerque CFG de, Maron-Gutierrez T, Silva AR, Neto HC de CF (2017). Role of Nitric Oxide Synthase in the Function of the Central Nervous System under Normal and Infectious Conditions. *Nitric Oxide Synthase - Simple Enzyme-Complex Roles.*, <https://doi.org/10.5772/67816>
- Réus GZ, Stringari RB, Ribeiro KF, Cipriano AL, Panizzutti BS, Stertz L, Lersch C, Kapczinski F, Quevedo J (2011). Maternal deprivation induces depressive-like behaviour and alters neurotrophin levels in the rat brain. *Neurochem. Res.*, 36: 460–466. <https://doi.org/10.1007/s11064-010-0364-3>
- Rhaimi S, Brikat S, Lamtai M, Ouhssine M (2023). Acute Oral Toxicity and Neurobehavioral Effects of Salvia officinalis Essential Oil in Female Wistar Rats. *Adv. Anim. Vet. Sci.*, 11(4): 654–662. <https://doi.org/10.17582/journal.aavs/2023/11.4.654.662>
- Salter M, Duffy C, Garthwaite J, Srijbos PJLM (1995). Substantial regional and hemispheric differences in brain nitric oxide synthase (NOS) inhibition following intracerebroventricular administration of N ω -nitro-L-arginine (L-NA) and its methyl ester (L-NAME). *Neuropharmacology.*, 34: 639–649. [https://doi.org/10.1016/0028-3908\(95\)00036-6](https://doi.org/10.1016/0028-3908(95)00036-6)
- Schwarz JM, Bilbo SD (2011). LPS elicits a much larger and broader inflammatory response than Escherichia coli infection within the hippocampus of neonatal rats. *Neuroscience Letters.*, 497: 110–115. <https://doi.org/10.1016/j.neulet.2011.04.042>
- Tomaz VS, Cordeiro RC, Costa AMN, De Lucena DF, Nobre Júnior H V., De Sousa FCF, Vasconcelos SMM, Vale ML, Quevedo J, Macêdo D (2014). Antidepressant-like effect of nitric oxide synthase inhibitors and sildenafil against lipopolysaccharide-induced depressive-like behavior in mice. *Neurosci.*, 268: 236–246. <https://doi.org/10.1016/j.neuroscience.2014.03.025>
- Zghari O, Azirar S, Lamtai M, El Hessni A, Ouichou A, Mesfioui A (2023). Intrahippocampal dose-dependent effects of aluminum injection on affective and cognitive response in male Wistar rat: potential role of oxidative stress. *Egypt. J. Basic Appl. Sci.*, 10: 460–475. <https://doi.org/10.1080/2314808x.2023.2229623>
- Ziaja M, Lubieniecka J, Lewicka M, Pyka J, Plonka PM (2011). Changes in nitric oxide content following injury to the neonatal rat brain. *Brain Res.*, 1367: 319–329. <https://doi.org/10.1016/j.brainres.2010.10.006>