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Stage-Specific Effects of Maternal Circadian Rhythm Disruption during Pre-Pregnancy, Pregnancy, and Lactation on Behavioral and Neurobiological Outcomes in Male Rat Offspring

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Abstract

Maternal circadian rhythm disruption (CRD) during reproduction is increasingly recognized as a risk factor for long-term neurobehavioral disturbances in offspring. However, the stage-specific consequences of maternal CRD remain poorly understood.This study investigated the effects of maternal CRD during distinct reproductive phases, pre pregnancy mother (PPM), pregnancy mother (PM), and lactationg mother (LM) on behavioral and molecular, and structural outcomes in male offspring.

Nulliparous Wistar rats were exposed to constant light (300 lux, 14 days) to induce CRD, while controls remained on a 12:12 h light/dark cycle. Male offspring were assessed for anxiety-like behavior (Elevated Plus Maze), depressive-like behavior (Forced Swim Test), hedonic behavior (Sucrose Preference Test), and recognition memory (Novel Object Recognition Test). Histological analysis of the hippocampal CA1 region and expression of Brain-Derived Neurotrophic Factor (BDNF) and PER2 were also evaluated.

PPM and PM offspring exhibited increased anxiety- and depressive-like behaviors, reduced birth weight, disorganized hippocampal CA1 cytoarchitecture, and molecular alterations, decreased BDNF expression in PM offspring and reduced PER2 expression in the suprachiasmatic nucleus (SCN) of PPM offspring, without impairments in hedonic or cognitive performance. In contrast, LM offspring displayed behavioral resilience, suggesting that postnatal circadian realignment may mitigate prenatal vulnerability.

Considering the functional and neurodevelopmental similarities between the third trimester of human pregnancy and the lactation period in rat, these findings underscore the clinical relevance of maintaining robust circadian regulation during the pre-pregnancy phase and the first-second trimesters, rather than focusing solely on the final trimester, to support optimal fetal brain development and overall health.

1. Introduction

Almost all vital physiological and metabolic processes are regulated by circadian rhythms, which are driven by cellular clocks distributed throughout the body [1]. These rhythms help synchronize brain and tissue functions with the external environment, allowing the body to perform distinct and often incompatible tasks appropriate to either day or night [2]. The central circadian clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is regulated by environmental cues known as zeitgebers especially light [3]. Light is the most powerful zeitgeber, resetting the SCN daily and aligning internal clocks with the 24-hour day. The ability of SCN cells to generate circadian rhythms arises from the transcriptional-translational activity of specific clock genes operating in interconnected positive and negative feedback loops [4]. Circadian rhythms control essential functions such as the sleep-wake cycle, hormone secretion, body temperature, and metabolism. Disruption of these functions can lead to sleep disorders, mood disturbances, metabolic issues, and impaired cognition [5]. Circadian rhythms begin to develop early in life, and their maturation is shaped by critical developmental windows. Many periconceptional challenges can influence not only pregnancy outcomes but also the long-term health of offspring through a process known as developmental programming, where early physiological stressors

alter fetal development to adapt postnatally [6]. In Wistar rats, the development of the suprachiasmatic nucleus (SCN) follows a well-defined temporal sequence. Neurogenesis within the SCN occurs during the embryonic period, whereas synaptogenesis predominantly takes place after birth. Circadian rhythms begin to emerge prenatally and continue to mature throughout the postnatal period, with stable entrainment and light responsiveness being fully established within the first few postnatal weeks. This maturational process is characterized by the antiphase expression of the core clock genes Per2 and Bmal1, along with rhythmic secretion of corticosterone, collectively indicating the early functional establishment of the circadian system [7,8]. Disruption of circadian rhythms during gestation or the perinatal period, such as exposure to constant light, leads to persistent behavioral and cognitive impairments, including anxiety, depression, and hippocampal dysfunction [9]. These adverse outcomes are strongly linked to alterations in brain-derived neurotrophic factor (BDNF), a key regulator of neuronal survival, differentiation, and synaptic plasticity [10]. Converging clinical and preclinical evidence implicates hippocampal BDNF in the pathophysiology of depression, demonstrating that stress-induced reductions in BDNF levels are closely correlated with depressive symptomatology and can be reversed by antidepressant treatment [11]. Moreover, circadian regulation of hippocampal BDNF expression has been documented, providing a mechanistic link between circadian rhythm disruption and mood-related neuropathology [12]. To summarize, research indicates that disruption of circadian rhythms can lead to behavioral and cognitive impairments, and such disturbances in mothers may also impact their offspring. Therefore, in this study, we aimed to investigate the effects of maternal circadian rhythm disruption before pregnancy, during pregnancy, and throughout lactation on behavioral, molecular, and molecular markers related to anxiety, depression, memory, and obsessive-compulsive traits in male rat offspring.

2. Materials and methods

2.1. Animals

Adult Wistar rats (50 females, 20 males; females: 260–280 g, males: 300–340 g; aged 14–16 weeks) were obtained from the Shahroud Institute (Semnan, Iran). Animals were acclimated for two weeks under standard conditions (12/12 h light/dark cycle, (lights on at 07:00 h and off at 19:00 h; temperature 24 ± 2 °C) with ad libitum access to food and water. All procedures were approved by the Institutional Care and Ethical Committee of Shahroud University of Medical Sciences (Ethics code: IR.SHMU.AEC.1403.005) and conducted in accordance with laboratory animal care guidelines. Clinical trial registration number not applicable.

2.2. Experimental design

The estrous cycle of nulliparous female rats was monitored by daily vaginal smears. Females in estrus were randomly paired with males (1:1 ratio) to allow individual monitoring. Mated females were then randomly assigned to four groups (n=10 per group):

- Control: Maintained under standard laboratory conditions (12/12 h light/dark cycle, ~ 300 lux white ambient illumination).

The other three groups were exposed to continuous light (24 h Light) at the same intensity as the control group, in the following order.

- Pre-pregnancy mother group (PPM): Exposed to constant light condition for two weeks before mating; females were then checked for estrous cycle and mated.
- Pregnancy mother group (PM): Exposed to constant light condition from day 0 of pregnancy (vaginal plug detection) until day 14 of gestation.
- Lactating mother group (LM): Exposed to constant light condition from delivery until day 14 postpartum together with their pups. The experimental procedure is illustrated schematically in Figure 1.

2.3. Vaginal Smear Test

In this study, determining the first day of pregnancy for the PM group was essential. Therefore, vaginal cytology was performed by preparing vaginal smears and analyzing cellular changes. The estrous cycle in rats lasts for approximately four days, and its stages can be identified based on the predominant cell types observed in vaginal smears. Vaginal smear samples were collected according to the method described by Yener et al. [13]. Briefly, a plastic pipette was filled with 200 µl of sterile saline and gently inserted about 5 mm into the vagina. The saline was flushed in and out two or three times to collect the vaginal lavage sample. The collected fluid was then placed on a glass slide and examined directly under a light microscope.

The stage of the estrous cycle was determined as follows: A predominance of nucleated epithelial cells indicated the proestrus phase. A predominance of irregular, cornified epithelial cells indicated the estrus phase. The presence of an approximately equal mix of leukocytes, cornified cells, and nucleated epithelial cells indicated the metestrus phase. A predominance of small, round leukocytes indicated the diestrus phase (Figure 2).

2.4. Behavioral Tests

Male offspring born to dams with disrupted circadian rhythms were subjected to behavioral assessments using a battery of standardized tests. From each maternal group, ten male offspring were selected and evaluated at eight weeks of age (approximately postnatal day 56), corresponding to early adulthood in rats.

2.4.1. Sucrose Preference Test (SPT)

The sucrose preference test was used to assess anhedonia as an indicator of depression-like behavior. Animals were first habituated to two drinking bottles one containing 2% sucrose solution and the other plain water—in their home cage for 48 hours prior to testing. On the test day, Rats were deprived of food

and water for 4 hours (from 8:00 am to 12:00 pm). Afterwards, they were given access to two identical bottles (one with 2% sucrose solution and one with water) for 1 hour. The bottles were weighed before and after the test period to determine fluid consumption. Sucrose preference was calculated using the following formula:

$$\text{Sucrose preference (\%)} = \frac{\textit{Weight of ingested sucrose solution}}{\textit{Weight of ingested sucrose solution} + \textit{Weight of ingested water}} \times 100$$

A decrease in sucrose preference indicates reduced sensitivity to reward (anhedonia) [14].

2.4.2. Marble Burying Test

The marble burying test was performed in transparent plastic cages (30 × 20 × 20 cm) containing a 5-cm layer of sawdust bedding. Twenty glass marbles (1.5 cm in diameter) were arranged evenly in a 4 × 5 grid on the bedding surface. Each rat was placed in the center of the cage and allowed to explore freely for 30 minutes. At the end of the session, the number of marbles buried (defined as being covered by bedding more than two-thirds of their surface) was recorded. The total number of buried marbles was used as an indicator of compulsive-like behavior [15].

2.4.3. Novel Object Recognition (NOR) Test

The novel object recognition (NOR) test was used to assess short-term memory in rats. Testing was conducted in a black Plexiglas arena (40 × 40 × 40 cm) under dim light (~15 lux). After a 5-minute habituation session, two identical objects fixed to the floor were presented for 5 minutes in the training phase. Following a 1-hour retention interval, one object was replaced with a novel object, and rats were allowed to explore for 5 minutes. Exploration was defined as the animal directing its nose within 2 cm of the object or touching it. The arena and objects were cleaned with 70% ethanol between trials. The percentage of time spent exploring the novel object and the discrimination index were calculated to evaluate recognition memory [16].

2.4.5. Elevated Plus Maze Test (EPM)

EPM test was employed to assess anxiety-like behavior and general locomotor activity. The apparatus consisted of a platform elevated 50 cm above the ground. Each animal was individually placed on the central platform and allowed to freely explore the maze for 5 minutes. Rat behavior during the test was recorded using a video camera and precisely analyzed with a computerized video tracking system [17]. The duration of time spent in the open arms and closed arms were also recorded.

2.4.6. Forced Swim Test (FST)

The Forced Swim Test (FST) was performed once at the experiment's end to assess depression-like behavior. Animals were individually placed in a cylindrical tank (35 cm height × 20 cm diameter) with 15 cm of 23–25°C water. After a 1-minute habituation, immobility was recorded for the subsequent 5 minutes of the 6-minute session using a video camera. Increased immobility indicated behavioral despair. Animals were dried and warmed post-test [18].

2.5. Tissue Preparation

Following completion of behavioral experiments, offspring rats were anesthetized with ketamine/xylazine (100/10 mg/kg, intraperitoneally). Under deep anesthesia, intracardiac perfusion was carried out using 120 mL of phosphate-buffered saline (0.01 M, pH 7.4), followed by 150 mL of 4% paraformaldehyde. The brain was then carefully removed and post-fixed in a 4% formaldehyde solution. Thereafter, the animals were euthanized by decapitation with a rodent guillotine in strict accordance with ethical guidelines. The skulls were then carefully opened with a scalpel, and the whole brains were rapidly extracted and post-fixed in 10% formalin for subsequent histological analysis. For molecular assays, including quantification of Per2 gene expression and BDNF protein levels in the SCN and hippocampus, freshly extracted brain tissues were homogenized in RIPA lysis buffer immediately after dissection.

2.5.1. Cresyl Violet (Nissl) Staining

Nissl staining was performed to evaluate neuronal loss in the hippocampus. Fixed brains were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin blocks. Serial coronal sections (7 μm thick) were cut using a sliding rotary microtome. Sections were deparaffinized in xylene (two changes, 10 minutes each), rehydrated through descending ethanol concentrations (100%, 95%, 70%), and rinsed in distilled water. Subsequently, sections were stained with 0.1% cresyl violet for 5–10 minutes, differentiated in 95% ethanol, dehydrated, cleared in xylene, and mounted with a permanent coverslip medium. Stained sections were examined under a light microscope, and neuronal counts was quantified using ImageJ software according to established Nissl assessment protocols [19].

2.6. Western blot analysis of BDNF

We evaluated BDNF protein levels in hippocampus tissue using Western blot. First, we homogenized hippocampal tissue in lysis buffer and centrifuged it for 30 minutes (4∘C, 11,000×g). We stored the supernatant at −20∘C and later determined the total protein concentration via the Bradford assay [20]. We then separated 60 μg of total protein from each sample by SDS-PAGE on 12.5% gels. Following transfer to polyvinylidene difluoride membranes, we blocked them in Tris-buffered saline with 0.01% Tween 20 (TBS-T) containing 3% BSA powder. Membranes were incubated overnight with a primary anti-BDNF antibody (Abcam, Cat No: ab108319, 1:500). After washing with TBS-T at room temperature, we incubated the blots for 90 minutes with a goat anti-rabbit IgG HRP-conjugated secondary antibody (Abcam, Cat No: ab6721, 1:1000). Antibody binding was detected using ECL substrate. To ensure equal loading, we subsequently incubated the membranes with a β-actin loading control antibody (Abcam, Cat

No: ab8227, 1:2500). Finally, we quantified the density of the protein bands on X-ray films using ImageJ software [21].

2.7. Quantitative Real-Time PCR Analysis (RT-qPCR)

Expression levels of the Per2 gene were quantified using quantitative real-time PCR (RT-qPCR). SCN tissues were immediately preserved in RNAlater solution and stored at -80°C until processing. Total RNA was extracted using RNX-plus solution (Sinaclon, Iran; Cat. No. EX6101) in accordance with the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and only samples with A260/A280 ratios between 1.8 and 2.0 were included for further analysis.

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the Easy cDNA Ultra™ Synthesis Kit (Parstous, Iran; Cat. No. A101161), following the manufacturer's protocol. RT-qPCR amplification was conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using RealQ Plus 2× Master Mix Green (Ampliqon, Denmark; Cat. No. A325402). Each 20 µL reaction mixture contained 2 µL cDNA, 10 µL 2× Master Mix Green, 0.5 µL each of forward and reverse primers (final concentration of 0.2 µM), and 7 µL nuclease-free water. The thermal cycling profile consisted of an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Primer specificity and amplification efficiency were confirmed by melting curve analysis. Relative expression levels of Per2 were normalized to the housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase (Hprt), which has been validated as a stable reference gene in circadian expression studies in mice [22]. Gene expression levels were calculated using the 2^−ΔΔCT method, and all reactions were performed in technical triplicates to ensure reproducibility and accuracy . The specific primers used are detailed in Table 1.

Table 1. Used primers in RT-qPCR.

Gene symbol	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'	PCR Product Size (bp)
Per2	CACCAAGTGACGGGTCGAG	CATTCATGTCGGGCTCTGGA	80
Hprt	CTCCTCAGACCGCTTTTCCC	TAATCACGACGCTGGGACT	71

Abbreviations: RT-qPCR, Quantitative Reverse Transcription Polymerase Chain Reaction; Per2, period circadian regulator 2; Hprt, hypoxanthine-guanine phosphoribosyl transferase.

2.8. Statistical Analysis

We assessed the normality of variables using the Shapiro–Wilk test. For comparisons of behavioral tests, RT-qPCR, Western blot analysis, and histological assessment, we used one-way ANOVA with Tukey's post-hoc test for parametric data and non-parametric equivalents for non-parametric data. We considered $P<0.05$ to be statistically significant. All statistical analyses were performed using GraphPad Prism software. Data are presented as mean ± S.E.M.

3. Results

3.1. Animal weight monitoring

In animals, body weight was used as an index of health. The results showed a significant difference in weight among the groups ($F(3,36) = 19.5$, $p < 0.001$). Specifically, the PPM and PM groups exhibited a significant reduction in weight compared to the control group, whereas no significant difference was observed between the LM and control groups. The results are presented in Table 2.

Table 2: Comparison of body weight (g) among groups

Groups	Control	PPM	PM	LM
Weight body	182.8±3.068	***129.2±7.621	**142.3±4.186	201.3±10.74

3.2. Sucrose preference, marble burying and novel object recognition test

Behavioral assessments, including the sucrose preference, marble burying, and novel object recognition tests, were conducted to evaluate anhedonia, compulsive-like behavior, and recognition memory, respectively. Analysis revealed no significant differences among the four groups (SPT: $F(3,36)=3.78$, $p=0.02$; MBT: $F(3,36)=0.0914$, $p=0.96$; NOR: $F(3,36)=0.326$, $p=0.81$; Figures 3A–C), suggesting that continuous light exposure during different stages of maternal pregnancy does not markedly affect these behavioral outcomes in the offspring.

3.5. Elevated plus maze test

ANOVA revealed highly significant main effects of group on time spent in the closed arm ($F (3,36) =28.5$, $p<0.001$; Figure 4A) and time spent in the open arm ($F (3,36) =17.3$, $p<0.001$; Figure 4B) of the Elevated Plus Maze. Post-hoc analyses for the closed arm showed significantly greater time spent by PPM and PM groups compared to Control ($p < 0.001$), and significantly less time spent by the LM group compared to both PPM and PM groups ($p< 0.001$). For the open arm, PPM and PM groups spent significantly less time compared to Control ($p < 0.01$), while the LM group spent significantly more time compared to both PPM and PM groups ($p< 0.001$).

3.6. Forced swimming test (FST)

ANOVA indicated a significant main effect of group on immobility time in the Forced Swim Test ($F(3,36)=8.62$, $p=<0.001$; Figure 5A). Further analysis revealed that both the PPM group and PM group exhibited significantly increased immobility times compared to the Control group. Specifically, the PPM group displayed a highly significant increase in immobility time relative to the Control group ($p< 0.001$). The PM group also showed a significant increase in immobility time compared to the Control group ($p< 0.01$). In contrast, LM group did not demonstrate a statistically significant difference in immobility time when compared to the Control group. These findings suggest heightened depression-like behavior in the PPM and PM groups.

ANOVA revealed a significant main effect of group on struggling time in the Forced Swim Test ($F(3,36) =6.02$, $p=0.002$; Figure 5B). Post-hoc comparisons indicated that both PPM group and PM group exhibited significantly lower struggling times compared to the Control group ($p< 0.05$). This suggests reduced active coping behavior in these groups. In contrast, the LM group did not show a statistically significant difference in struggling time when compared to the Control group. These findings indicate altered behavioral responses to stress in the PPM and PM groups.

ANOVA revealed no significant main effect of group on swimming time in the Forced Swim Test ($F(3,36)=0.668$, $p=0.58$; Figure 5C). This indicates that there were no statistically significant differences in swimming behavior among the Control, PPM, PM, and LM groups.

3.7. Hippocampal histology

Histological evaluation of the hippocampal CA1 region using Nissl staining revealed significant differences among the groups ($F(3,16) =8$, $p=0.001$). In the PPM and PM groups, disruption and disorganization of the pyramidal cell layer were observed, with a noticeable separation of CA1 layers and the presence of several dark neurons, indicating neuronal stress and potential early degenerative changes.

In contrast, the LM group showed a cellular arrangement comparable to the control group. Quantitative analysis confirmed that the number of dark neurons was significantly higher in the PPM group compared to the control, PM, and LM groups, while no significant difference was observed between the LM and control groups (Figure 6E).

3.8. Hippocampal BDNF protein

ANOVA revealed a highly significant main effect of group on hippocampal BDNF protein levels ($F(3,8) =16.4$, $p<0.001$; Figure 7). Post-hoc analysis indicated that PM group exhibited a highly significant decrease in BDNF/β-actin ratio compared to the Control group ($p< 0.001$). While PPM group showed a slightly lower mean BDNF level than the Control group, this difference was not marked as statistically significant in the figure. LM group also displayed BDNF levels lower than the Control and PPM groups, but higher than the PM group, though no specific statistical comparisons are indicated for LM against Control or PPM.

3.9. Quantitative Reverse Transcription Polymerase Chain Reaction (RT- qPCR) in SCN

Analysis of variance (ANOVA) revealed a robust and statistically significant main effect of group on *Per2* gene expression fold change within SCN ($F(3,8) = 88.9$, $p<0.001$; Figure 8). PPM group showed a highly significant decrease in gene expression compared to the Control group ($p<0.001$). PM group also exhibited a significant decrease compared to the Control group ($p<0.05$). Conversely, the LM group displayed a highly significant increase in gene expression compared to the Control group ($p<0.001$). Furthermore, the LM group also showed a highly significant increase compared to both the PPM and PM groups ($p<0.001$).

4. Discussion

This study aimed to investigate the long-term behavioral and molecular consequences of maternal circadian rhythm disruption (CRD) during three different stages on male offspring. The findings revealed that the timing and duration of maternal CRD differentially influence offspring outcomes, affecting affective behaviors, neuronal integrity, and specific gene expression profiles, while leaving other domains, such as cognitive function and hedonic behavior, largely intact.

Our results demonstrate a clear dissociation between cognitive/hedonic function and affective behaviors. Specifically, while constant light exposure for 14 days did not significantly alter performance in sucrose preference, marble burying, or novel object recognition (NOR) tests across all groups (PPM, PM, and LM), the prenatally misaligned (PM) and pre- and postnatally misaligned (PPM) groups exhibited significantly heightened anxiety-like and depressive-like behaviors. This was evidenced by increased immobility in the forced swim test (FST) and more time spent in the closed arms of the elevated plus maze (EPM). This divergence suggests that the brain systems supporting cognitive functions, such as the hippocampus, may not be the primary target for developmental changes caused by maternal CRD during this period. As noted by Bayer et al. [23] and Roman and Karlsson [24], the delayed maturation of granule cells in the dentate gyrus and the protracted development of hippocampal-dependent cognitive behaviors may allow for compensatory mechanisms to reverse potential negative effects induced by constant light conditions.

The observed increase in anxiety-like and depressive-like behaviors is consistent with prior studies that have linked early-life circadian disruption to heightened affective disorders [25,26]. The findings partially align with previous reports [27,9], which also observed that chronic or early postnatal circadian disruption induces anxiety-like behavior and alters stress reactivity. However, our protocol, which involved moderate, gestation-only phase shifts, was less intense and shorter in duration than the prolonged exposure used in some previous studies, such as the one by Zhang et al.[27]. This protocol-specific difference may explain why we did not observe significant cognitive deficits, suggesting that a limited exposure window may allow for physiological compensation that prevents severe behavioral phenotypes from emerging.

Histological analysis of the CA1 region of the hippocampus confirmed a higher density of dark neurons and disorganization of neuronal layers in the PM and PPM groups. This disorganization and loss of neuronal integrity likely underlie some of the observed behavioral changes. This is consistent with our findings that the downregulation of brain-derived neurotrophic factor (BDNF), a key molecule for synaptic plasticity and neuronal survival, was significantly reduced in the PM group and showed a non-significant downward trend in the PPM group. As previous studies have demonstrated [28,29] circadian disruption is associated with the downregulation of neuroplasticity-related pathways. The reduced BDNF expression and CA1 layer disorganization, despite the absence of cognitive deficits, suggest a silent neurobiological vulnerability that may only manifest under additional stress or challenges. This dissociation between molecular changes and overt behavioral deficits aligns with developmental programming frameworks [30]. The observed changes in neuronal integrity and BDNF expression in the PM and PPM groups, along with the absence of these effects in the LM group, this highlights the prenatal period as a particularly sensitive window for developmental programming. Consistent with current understanding, brain development especially the maturation of the suprachiasmatic nucleus (SCN) takes place during the embryonic and early postnatal stages. Notably, this timeline applies to rodents, in which the lactation period corresponds physiologically to the third trimester of human gestation. Therefore, these findings suggest that in humans, circadian disruption during the third trimester may exert milder effects on offspring anxiety and depression than disturbances occurring earlier in or even prior to pregnancy [6].

The study revealed differential effects on the expression of the circadian regulator gene, PER2, in SCN. The observed stage-dependent modulation indicates that reproductive state critically alters the molecular responsiveness of the central circadian pacemaker to photic disruption. The attenuation of PER2 expression in the PPM group suggests that constant light suppresses the transcriptional activity of the SCN clock, likely through disturbed photic signaling to core SCN neurons and reduced activation of intracellular pathways downstream of CREB and CLOCK/BMAL1. PM group, the maintenance of stable PER2 levels points to a buffering effect of gestational hormones, which may modulate synaptic inputs or intracellular signaling cascades within the SCN, thereby preserving circadian gene oscillations. The elevated PER2 expression during LM group implies enhanced photic or homeostatic drive on SCN neurons, potentially mediated by prolactin- or oxytocin-dependent modulation of GABAergic and glutamatergic transmission.

Previous studies [31], have shown that constant light exposure can dysregulate the HPA axis. The observed increase in anxiety-like and depressive-like behaviors may be linked to this dysregulation, potentially via altered levels of glucocorticoids like corticosterone, a known consequence of stress and circadian disruption [32,33]. The decrease in PER2 expression in the PPM group suggests that sustained CRD, both prenatally and postnatally, has a more profound effect on the core clock machinery itself, which could explain the more pronounced behavioral deficits in this group.

Interestingly, the LM group, despite experiencing CRD, demonstrated a more resilient and exploratory behavioral profile. As discussed above, research has shown that the brain growth spurt in humans, which occurs during the third trimester, is equivalent to the first two weeks after birth in rodents [34]. This

period represents a critical window of neurodevelopment and vulnerability to environmental disruptions. Given that key neurodevelopmental processes such as neuronal proliferation and migration occur during gestation and early postnatal life, maternal circadian disturbances may causally influence offspring brain function by altering BDNF levels, Per2 gene expression, and SCN morphology. The findings may offer insights into how disrupted maternal circadian timing contributes to neuropsychiatric vulnerability in offspring, with potential translational relevance for improving maternal health practices and early interventions in human perinatal care.

Our findings in line with previous studies show that maternal exposure to 300 lux light before and during pregnancy led to a significant reduction in infant body weight [35]. This effect was not observed when constant light exposure occurred during the lactation period. This underscores the prenatal phase as a more critical window of susceptibility for growth-related outcomes. The inconsistency of constant light effects on fetal growth reported in the literature [35,36] highlights the importance of light intensity, species differences, and exposure timing. Our results, in line with Gozeri et al, support the hypothesis that even a moderate level of light, comparable to daily environmental conditions, can significantly impact fetal development [37], The reduction in fetal weight might be linked to the downregulation of BDNF, which has been shown to modulate fetal development, particularly with regard to weight and the maturation of fetal organs [38]. The presence of BDNF in the placenta suggests a crucial role in placental function and nutrient transfer [39], which could be impacted by CRD.

The collective findings of this study have significant clinical relevance, particularly for human populations exposed to CRD, such as shift workers. The results indicate that maternal CRD, even at moderate levels, can have lasting and detrimental effects on the offspring's emotional well-being and neurobiological integrity. The heightened anxiety- and depressive-like behaviors observed in the offspring parallel the increased prevalence of mood disorders linked to early-life circadian misalignment [40]. This suggests that pregnant individuals who engage in shift work or are otherwise exposed to chronic CRD may have children who are at a higher risk for developing affective disorders.

While we found no significant differences in cognitive or hedonic behaviors, the presence of underlying molecular and structural changes, such as reduced BDNF and CA1 layer disorganization, suggests a latent vulnerability. Future studies should investigate whether these "silent" vulnerabilities can be unmasked by additional stressors later in life, such as social defeat or chronic stress, which could reveal a greater susceptibility to psychiatric disorders. Additionally, future research should explore the specific hormonal and epigenetic mechanisms that link maternal CRD to these long-term offspring outcomes, particularly the role of melatonin, glucocorticoids, and sex-specific differences.

The finding that the prenatal period represents a critical window of heightened vulnerability for offspring emotional development, neurobiological integrity, and even physical growth (as reflected by reduced weight) suggests that pregnant mothers—especially those engaged in shift work or exposed to other circadian rhythm–disrupting conditions—may require greater rest and support during the early and middle stages of gestation or even pre pregnancy duration. Given that the most pronounced effects (on

anxiety, depression, and BDNF levels) resulted from prenatal CRD exposure, while the LM (Late Misalignment) group—representing late-gestation or postnatal exposure—exhibited greater resilience. Therefore, pregnant individuals working in shift-based or irregular schedules might benefit from reduced circadian disruption—through adjusted work hours or earlier maternity leave—during the pre-pregnancy and first- second trimesters to help prevent long-term affective and neurobiological vulnerabilities in their offspring.

In conclusion, this study provides strong evidence that maternal CRD during different satges acts as a significant developmental stressor, selectively impacting the emotional and neurobiological development of male offspring. The findings emphasize that the timing of CRD is a critical determinant of its effects and that the prenatal phase represents a period of heightened susceptibility for both growth and emotional development.

Declarations

Funding Declaration

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Author contributions

Yeganeh Derakhshan contributed to in vivo experiments, acquisition of data, analysis of data, and writing the original draft of manuscript.

Behzad Garmabi contributed to conception and design, analysis and interpretation of data, contributed to the supervision and acquired the funding, revisiting the manuscript critically, and approval of the final version.

Shima Mohammadi contributed to acquisition of data, analysis of data and writing the original draft of manuscript and editing.

Amir Atashi contributed to RT-qPCR experiments, analyzed the molecular data, and contributed to writing the final draft of manuscript.

Parham Reisi contributed to analysis of data, review, and editing the manuscript.

Seyed Davar Siadat contributed to approval of the final version.

Declaration of interest

The authors declare that they have no conflict of interest.

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Figures

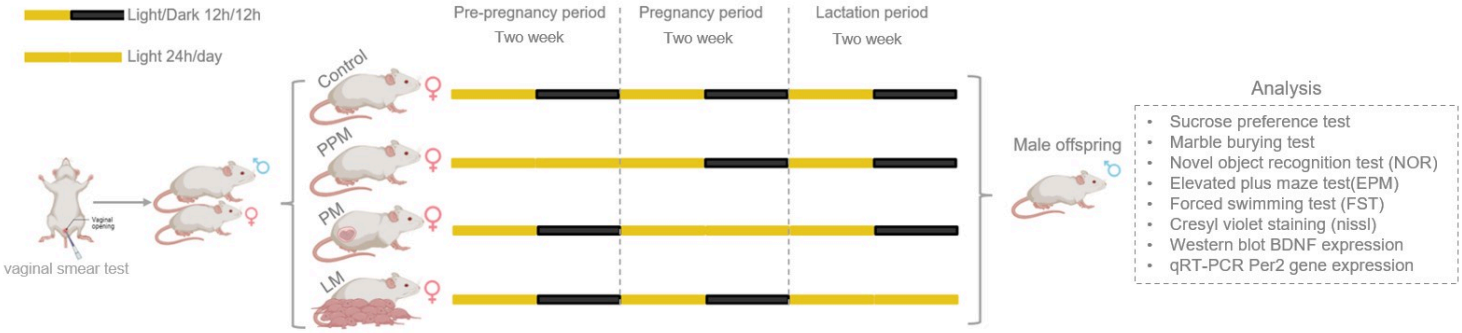

Figure 1

Experimental protocol for maternal circadian rhythm disruption. Control females were maintained under standard laboratory conditions (12 h/12 h light/dark cycle, ~300 lux). In the pre-pregnancy mother group (PPM), females were exposed to constant light (~300 lux) for two weeks prior to mating, after estrous cycle confirmation. In the pregnancy mother group (PM), females were exposed to constant light from day 0 of pregnancy (vaginal plug detection) to gestational day 14. In the lactating mother group (LM), females and their pups were exposed to constant light from delivery to postpartum day 14. After light manipulation, all groups were returned to standard 12 h/12 h conditions, and male offspring were subjected to behavioral, histological, and molecular analyses.

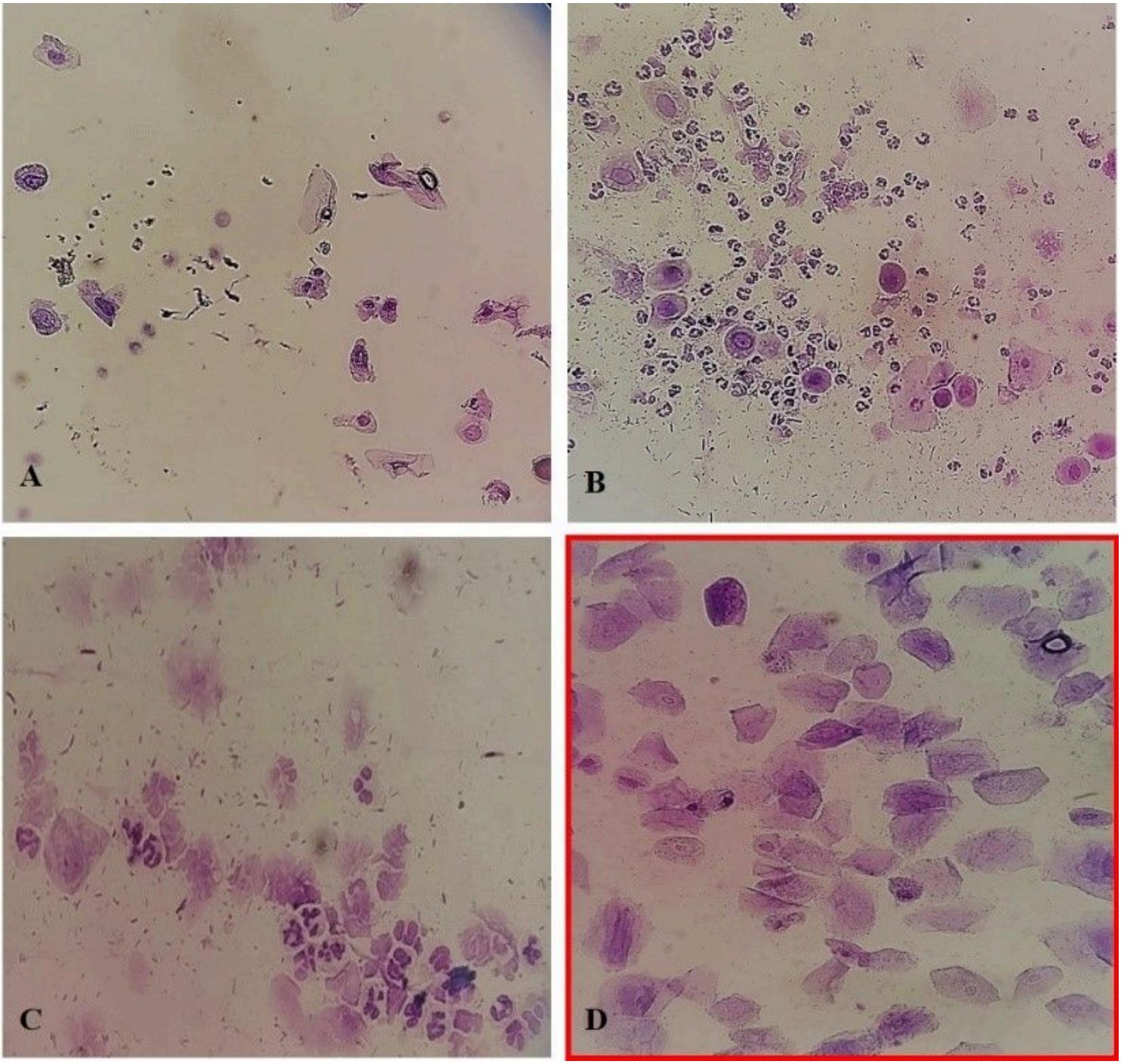

Figure 2

Representative photomicrographs of vaginal smear cytology showing the different stages of the estrous cycle in rats. (A) Proestrus phase: predominance of nucleated epithelial cells. (B) Diestrus phase: predominance of small, round leukocytes. (C) Metestrus phase: presence of mixed cell types including leukocytes, cornified, and nucleated epithelial cells. (D) Estrus phase: predominance of large, irregular cornified epithelial cells without nuclei, which are indicated in the figure by a red box.

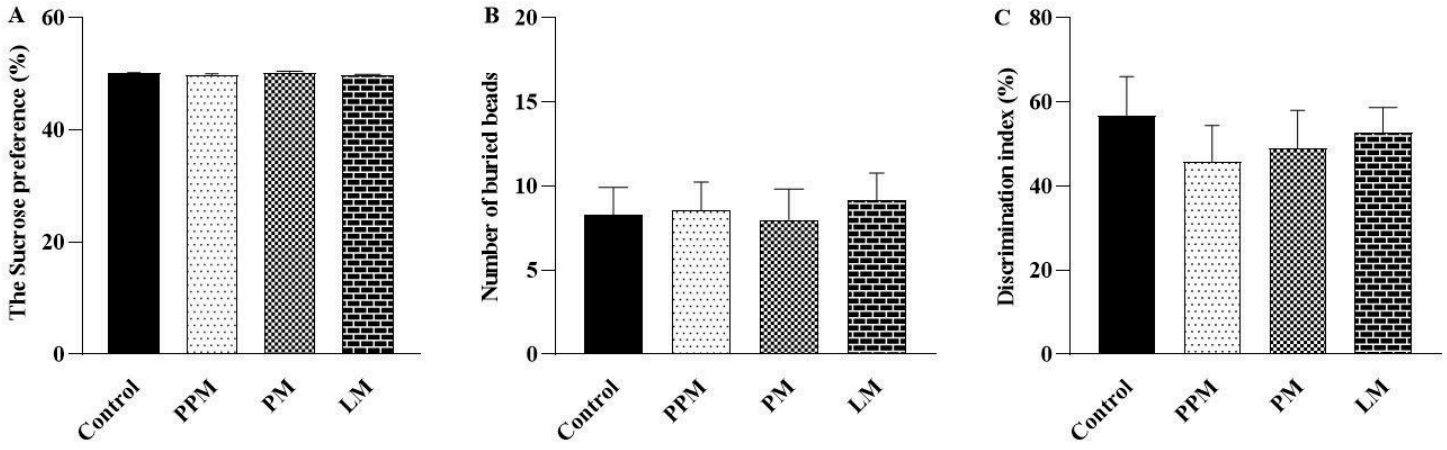

Figure 3

Behavioral performance of male offspring in (A) the sucrose preference test, (B) the marble burying test, and (C) the novel object recognition test in Control, Pre-pregnancy mother (PPM), Pregnancy mother (PM), and Lactating mother (LM) groups. No significant differences were observed among the groups. Data are presented as mean ± SEM (n=10 per group).

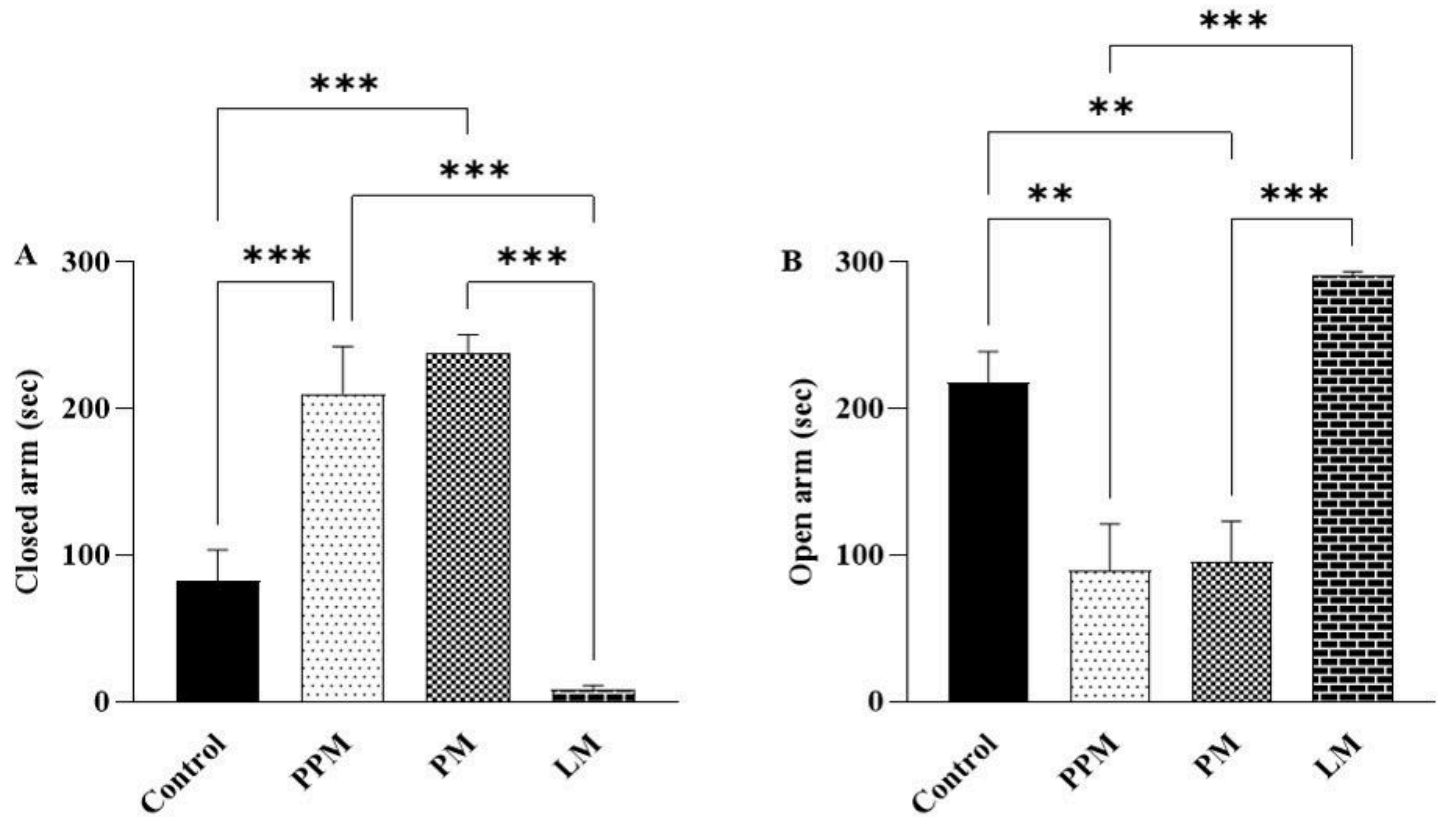

Figure 4

Elevated Plus Maze (EPM) behavioral parameters. (A) Time spent in the closed arm (seconds) by Control, Pre-pregnancy mother (PPM), Pregnancy mother (PM), and Lactating mother (LM) groups. (B)

Time spent in the open arm (seconds) by Control, PPM, PM, and LM groups. Data are expressed as mean ± SEM (n=10 animals per group). **p< 0.01, ***p<0.001 compared to the control groups.

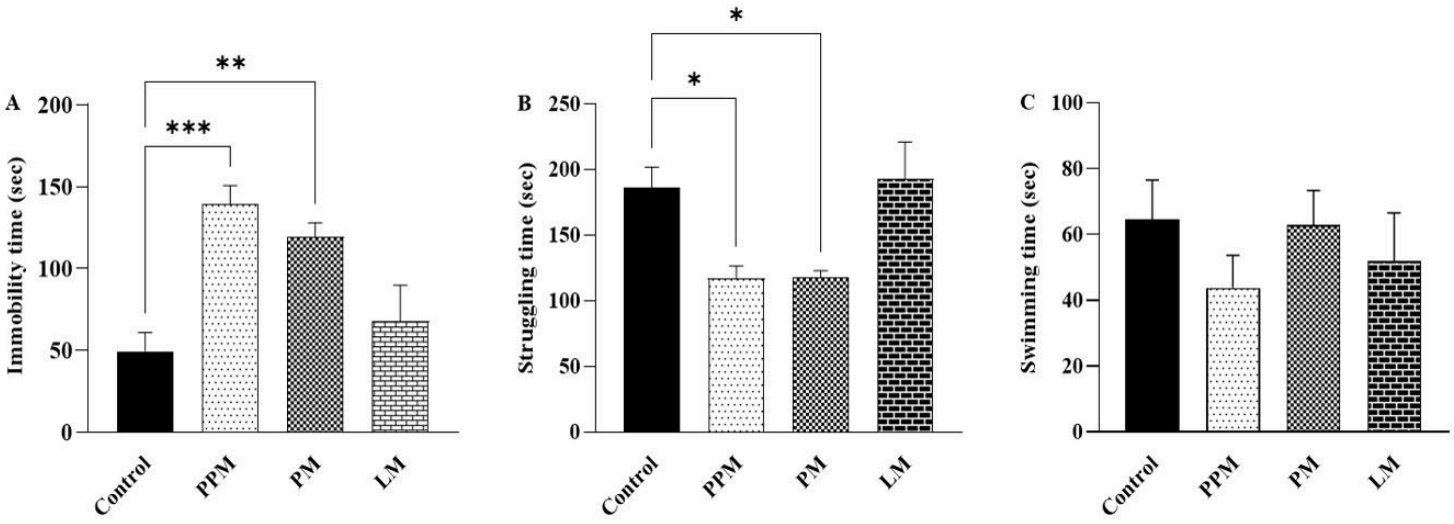

Figure 5

Behavioral parameters in the Forced Swim Test (FST). (A) Immobility time (seconds) in Control, Pre-pregnancy mother (PPM), Pregnancy mother (PM), and Lactating mother (LM) groups. A highly significant increase in immobility was observed in the PPM group compared to Control (***p< 0.001), and a significant increase was also seen in the PM group compared to Control (**p< 0.01). (B) Struggling time (seconds) in Control, PPM, PM, and LM groups. Both PPM and PM groups showed significantly reduced struggling time compared to Control (*p< 0.05). (C) Swimming time (seconds) in Control, PPM, PM, and LM groups, with no significant differences observed between groups. For all panels, n=10 animals per group. Data are expressed as mean ± SEM.

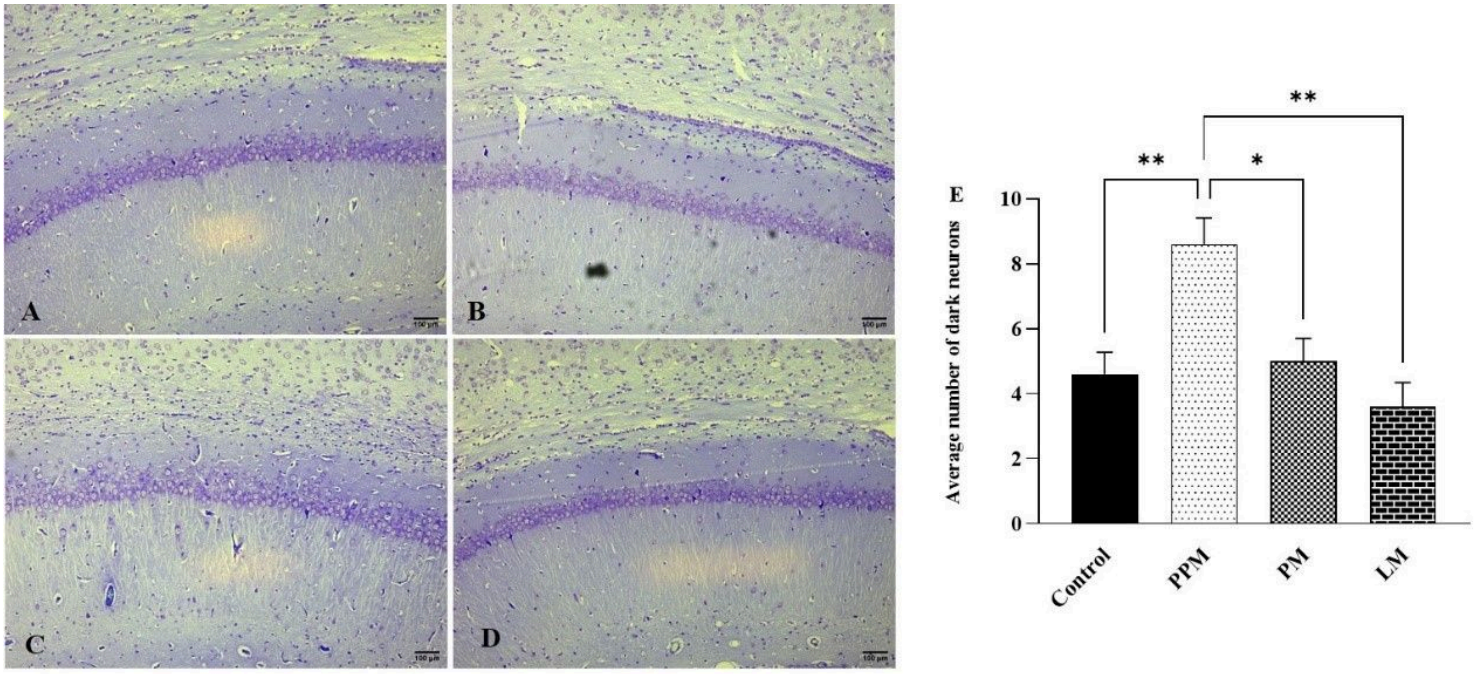

Figure 6

Nissl-stained sections of the hippocampal CA1 region in different groups: (A) Control, (B) Pre-pregnancy mother (PPM), (C) Pregnancy mother (PM), and (D) Lactating mother (LM). In the PPM and PM groups, disorganization of the pyramidal layer, separation of CA1 layers, and the presence of dark neurons are evident, whereas the LM group shows a cellular pattern similar to the control group. (E) Quantitative analysis of the average number of dark neurons in CA1. Data are presented as mean ± SEM. *p< 0.05, **p< 0.01 compared to the control groups. Scale bars: 100 μm, magnification200x.

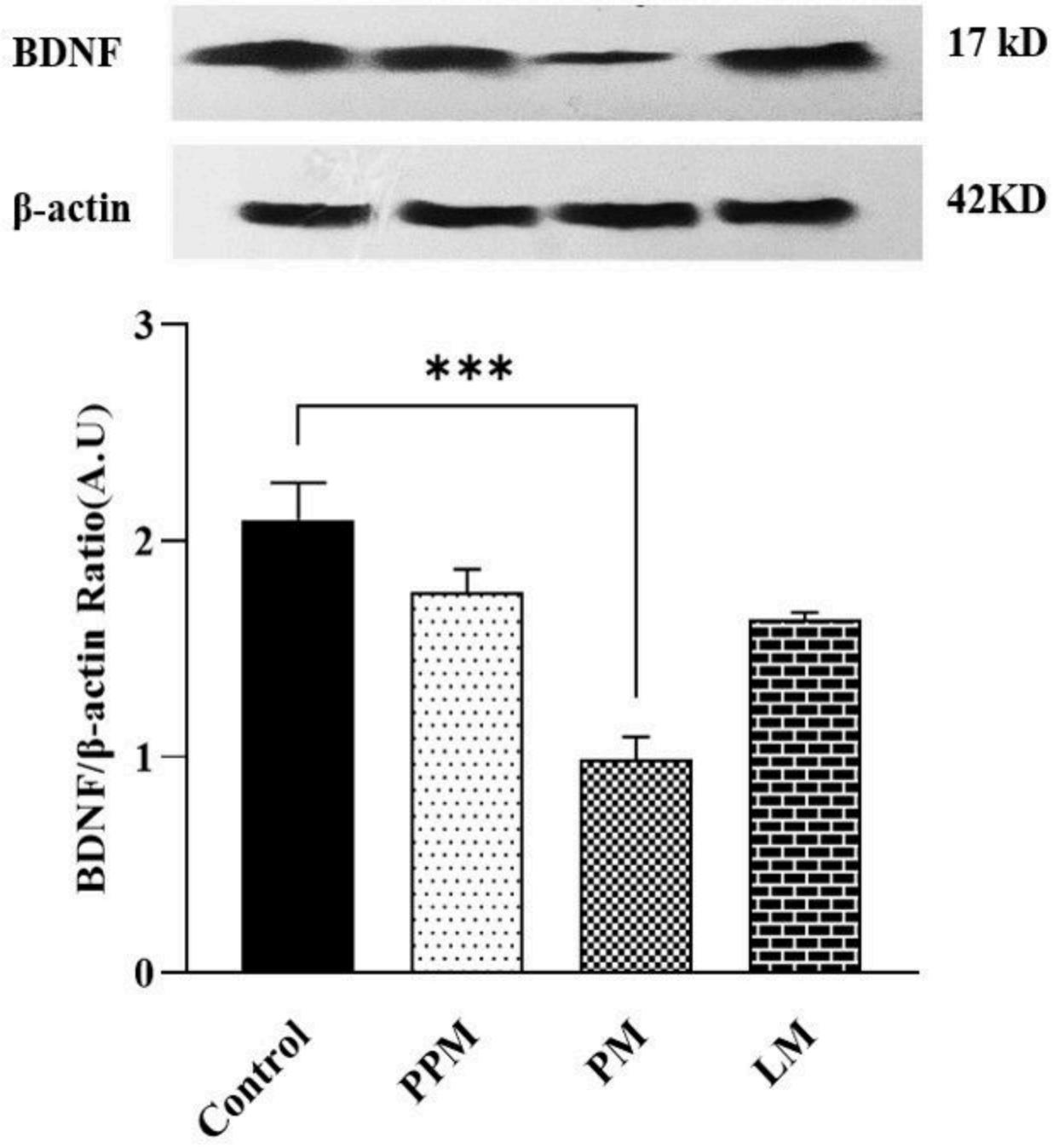

Figure 7

Representative Western blot bands (top panel) and quantification of BDNF protein expression (bottom panel). Hippocampal BDNF/β-actin protein ratio (A.U.) in Control, Pre-pregnancy mother (PPM), Pregnancy mother (PM), and Lactating mother (LM) groups. Data are expressed as mean ± SEM (n=3). A highly significant difference (***p< 0.001) was observed between the Control and PM groups.

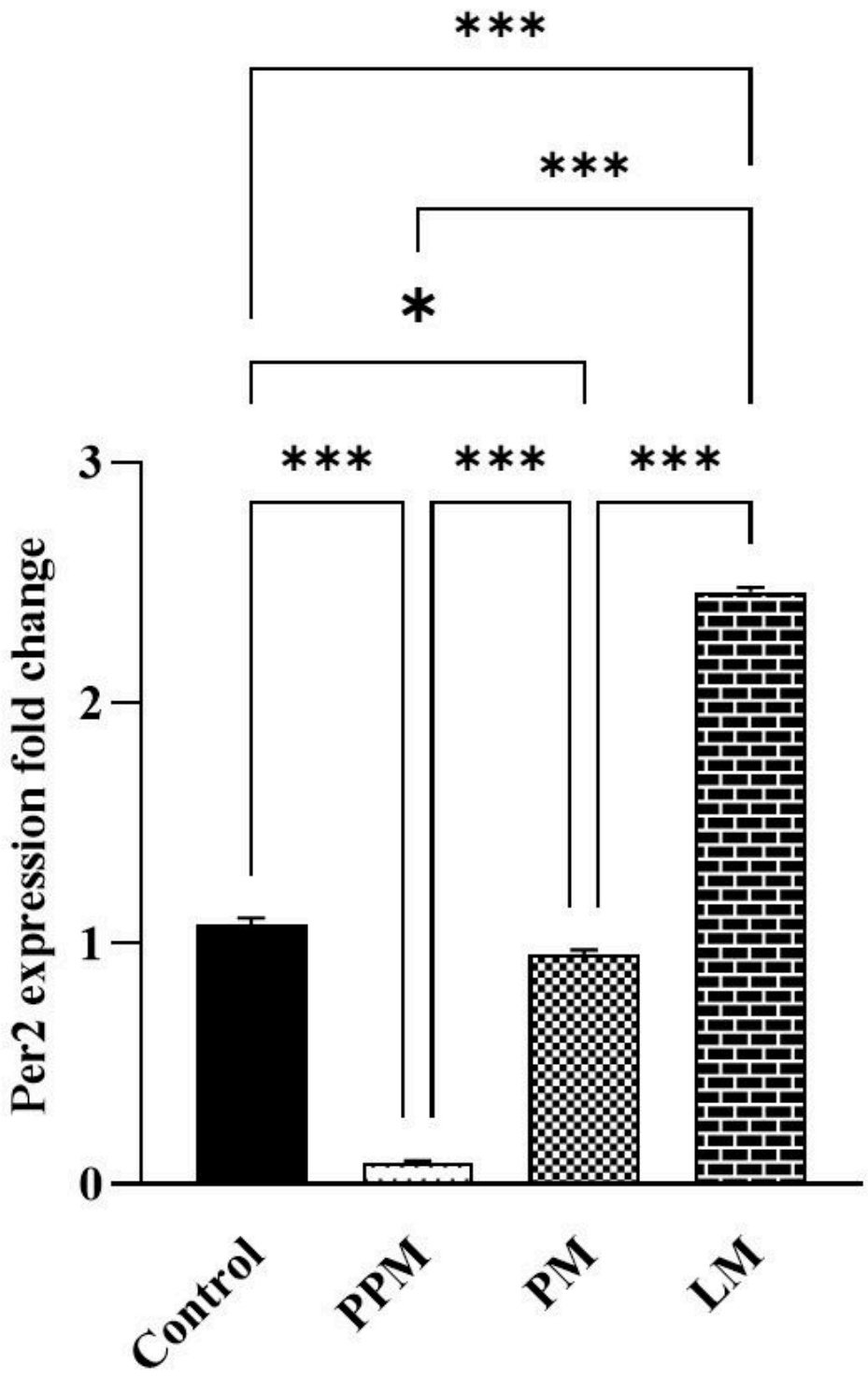

Figure 8

SCN Per2 expression fold change in Control, Pre-pregnancy mother (PPM), Pregnancy mother (PM), and Lactating mother (LM) groups. A significant decrease was observed in PPM and PM groups compared to Control (***p<0.001 and *p<0.05). Conversely, LM group showed a highly significant increased compared to Control (***p<0.001), and compared to both PPM and PM groups (***p<0.001). Data are expressed as mean ± SEM (n=3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Bactinp1.jpg
- Bactinp2.jpg
- BDNFP1.jpg
- BDNFP2.jpg