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Early Social Isolation Results Anxiety- and Depression-Like Behaviors and Neuroinflammatory Responses in Rats*

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ABSTRACT

Exposure to stress during early developmental stages adversely affects brain development and is associated with psychiatric conditions such as depression and anxiety. In this study, we investigated behavioral changes and levels of oxidative stress and inflammatory mediators in the prefrontal cortex (PFC) of young male Wistar rats subjected to 6 weeks of chronic social isolation. Sixteen male Wistar Albino rats were assigned to either a control group or a social isolation group. Animals in the isolation group were housed individually for six weeks. Depressive-like behavior and anxiety-like behavior were assessed using the forced swimming test (FST) and the open field test (OFT), respectively. Toll like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), glycogen synthase kinase 3 beta (GSK-3β), brain derived neurotrophic factor (BDNF), malondialdehyde (MDA), and reduced glutathione (GSH) levels in the prefrontal cortex were measured. Group differences were analyzed using the Mann–Whitney U test. In the social isolation group, time spent in the center zone during the OFT was reduced, while time in the periphery was significantly increased compared to controls, indicating elevated anxiety-like behavior. Socially isolated rats also exhibited greater immobility in the FST, reflecting depression-like behavior, and showed elevated levels of TLR4, NF-κB, GSK-3β, and MDA. No statistically significant differences were observed between the groups in BDNF and GSH levels. Behavioral disturbances induced by early-life social isolation stress may involve increased oxidative stress in the PFC and activation of the NF-κB/TLR4/GSK-3β signaling pathway.

Keywords: Anxiety, Depression, Inflammation, Prefrontal cortex, Social isolation.

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Introduction

Early-life social deprivation has detrimental effects on brain development and behavior, thereby increasing vulnerability to neuropsychiatric disorders (Chmelova et al., 2019; Kamaei et al., 2024). Long before the year 2020, mental illnesses constituted a significant component of the global disease burden, particularly depressive and anxiety-related conditions. In 2020, the COVID-19 pandemic was linked to a marked rise in the global prevalence of mental health disorders, with major depressive disorder increasing by 27.6% and anxiety disorders by 25.6% (Jiang et al., 2024). Post-weaning social isolation (SI), initiated at postnatal day 21, has been extensively employed as an experimental model to investigate the neurobiological mechanisms underlying depression (Toth et al., 2011). Animals subjected to social isolation display a range of depression- and anxiety-related

alterations (Dávila-Hernández et al., 2021; Dimonte et al., 2023), including heightened inflammatory activity and impaired neuroplastic processes. These outcomes may result from impaired connectivity and dysfunction of neural circuits in the brain during critical developmental stages (Kim et al., 2025).

Brain-derived neurotrophic factor (BDNF) has received considerable attention in neuroscience research because of its fundamental contribution to cognitive and behavioral processes within the central nervous system (Colucci-D'Amato et al., 2020). Notably, diminished BDNF expression has been widely documented in various neuropsychiatric disorders and cognitive deficits. Moreover, alterations in the BDNF system across specific brain regions are well established as markers of stress, even in socially deprived conditions. Together, these observations highlight BDNF's essential contribution to the brain's maturational processes following early-life social isolation stress (Di Trapano et al., 2025).

Accumulating evidence indicates that neuroinflammation plays a central role in the pathophysiology of depression, potentially serving as a primary causal mechanism (Leonard, 2018). Toll-like receptors (TLRs), members of the germline-encoded pattern recognition receptor family, have been identified as key mediators of inflammatory pathways implicated in depression, initiating immune responses. Clinical studies have reported elevated TLR4 mRNA and protein expression, along with increased nuclear factor kappa B (NF-κB) levels, in newly diagnosed major depression, suggesting that stress-induced neuroinflammation mediated via the TLR4 pathway may contribute to the disorder (Nie et al., 2018). The TLR4/NF-κB pathway represents a critical regulatory mechanism in inflammation, as its activation drives the expression of downstream pro-inflammatory cytokines (Kamaei et al., 2024; Liu et al., 2024; Weber et al 2013). Pro-inflammatory cytokines can suppress the production of BDNF, a key molecule fundamental for neuronal survival, differentiation, morphogenesis, and synaptic plasticity (Abu-Elfotuh et al., 2023, Kamaei et al., 2024).

Glycogen synthase kinase-3 beta (GSK-3β), a serine/threonine kinase, functions as a key regulatory node in multiple signaling networks. Disturbances in GSK-3β signaling have been linked to the development of a wide spectrum of psychiatric and neurological disorders, including schizophrenia, major depressive disorder, bipolar disorder, and neurodegenerative pathologies (Gholami-Zanjanbar et al., 2024). Oxidative stress, arising from excessive reactive oxygen species and impaired antioxidant defenses, is a key driver of neuronal injury and neuroinflammation, contributing to the pathogenesis of neurological disorders. Owing to its high metabolic activity and limited antioxidant capacity, the brain is particularly susceptible to oxidative damage. This study aimed to (i) examine the effects of early-life social isolation on anxiety- and depressive-like behaviors in adult male rats and (ii) elucidate the underlying molecular mechanisms, focusing on neuroinflammatory responses, oxidative stress, and BDNF alterations in the prefrontal cortex, a key region implicated in anxiety and depression.

Methods

Animals

Sixteen male Wistar albino rats, weaned on postnatal day 21, were included in the present study. The animals were supplied by the Experimental Animal Facility of Zonguldak Bülent Ecevit University. Throughout the experiment, rats were maintained under controlled environmental conditions (22–25 °C) with a 12-h light/dark cycle. Animals were assigned to either an individual housing condition (social isolation; n = 8) or a group housing condition (four rats per cage; n = 8). The sample size was selected based on previous studies (Pitcairn et al., 2024; Toth et al., 2011) using similar methodologies and in accordance with ethical considerations under the 3R principles.

Housing was provided in opaque plastic cages equipped with bedding material and metal grid tops. All experimental procedures complied with internationally accepted ethical standards for animal research and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of Zonguldak Bülent Ecevit University (Date: September 12, 2019, Approval number: 2019-15-12/09).

Beginning on postnatal day 21, rats assigned to the social isolation group were subjected to 6 weeks of individual housing. For this purpose, each rat was placed in a

separate cage, which was then covered and relocated to a different room to prevent visual and physical contact with other animals. Throughout the study, rats were maintained under the same care conditions and provided with fresh tap water and standard rat pellets, changed daily (Turan & Sayan Özaçmak, 2025).

Behavioral tests were conducted between 10:00 and 16:00 under standard lighting and temperature-controlled conditions. On the final day of the 6-week social isolation period, anxiety-like behavior was assessed using the open field test, and depression-like behavior was evaluated using the forced swim test. After behavioral assessments, animals were euthanized under high-dose anesthesia, and brain tissues were collected. The prefrontal cortex was dissected, and levels of TLR4, NF-κB, BDNF, and GSK-3β were measured using enzyme-linked immunosorbent assay (ELISA). Additionally, malondialdehyde (MDA), a marker of lipid peroxidation, and reduced glutathione (GSH), an indicator of antioxidant status, were quantified spectrophotometrically.

Behavioral Tests

On the test day, the Open Field Test was conducted prior to the Forced Swim Test to minimize the potential impact of stress induced by FST on locomotor and exploratory behavior.

Forced Swimming Test (FST)

Transparent and durable glass tanks (45 cm in height × 20 cm in diameter) were filled with water to a depth of 15 cm, maintained at 24–30 °C. A quiet room was selected to minimize stress and prevent exposure to light and noise. During the Forced Swim Test (FST), barriers were placed in front of the tanks to prevent animals from observing the tests of other rats. The FST was recorded using a camera. Each rat was placed in the water for 5 minutes, and immobility time was recorded. After the test, animals were gently dried with a towel and placed in a warm environment to allow complete drying. Immobility duration was used as an indicator of depression-like behavior (Erdem et al., 2024).

Open Field Test (OFT)

The open field test (OFT), developed by Walsh and Cummins (1976), is a widely used method to assess anxiety-like behavior in rodents. Locomotor activity and anxiety were measured based on line crossings, rearing, central square entries, and time spent in the center. Each rat was placed in the center of an 80 × 80 × 30 cm plexiglass arena, and its movements were recorded for 5 minutes. The apparatus was cleaned with 20% alcohol between subjects, and behavioral parameters were analyzed from video recordings by independent observers (Erdem et al., 2024).

Enzyme-linked immune sorbent assay (ELISA)

Brain tissues were homogenized in phosphate-buffered saline (pH 7.4) and centrifuged (3,000 g, 20 min, 4 °C). Supernatants were analyzed using ELISA kits for BDNF (Cloud-Clone Corp., SEA011Ra, USA), TLR4 (Sunbiore, China), NFkappaB (Sunbiored, China), and GSK3 beta (Sunbiored, China) according to the manufacturers' instructions, and results were read based on standard curves. Tissue samples were homogenized in 9 volumes of PBS (1:10, w/v). ELISA concentrations were calculated from the standard curve and are reported as pg/mL of tissue homogenate.

Biochemical Analyses

Malondialdehyde (MDA) levels were measured according to Casini et al. (1986). Homogenates were centrifuged (3,000 g, 15 min, 20 °C), and the supernatant was mixed with 1% butylated hydroxytoluene (BHT) and 0.67% thiobarbituric acid (TBA). Samples were boiled for 15 min, and absorbance was read spectrophotometrically at 535 nm (Casini et al., 1986). GSH levels were determined according to Aykaç et al. (1985). Supernatants were centrifuged (3,000 g, 8 min, 20 °C) in microcentrifuge tubes, and 1 mL of 0.3 M Na_2HPO_4 and 125 µL of dithiobisnitrobenzoate were added. Absorbance was measured spectrophotometrically at 412 nm.

Statistical Analyses

All data analyses were conducted using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). Data are presented as median (interquartile range, Q1-Q3). Group differences were assessed using the Mann–Whitney U test, and $p < .05$ was considered statistically significant.

Results

Body Weight Changes

Changes in body weight after 6 weeks of social isolation are shown in Table 1. Rats subjected to social isolation exhibited a significant increase in body weight compared to the control group ($p < .05$).

Table 1.

Changes in body weight after 6 weeks of social isolation and behavioral outcomes in the open field test (OFT) and forced swim test (FST). Data are presented as median (Q1–Q3) (n = 8 per group).

Parameters	Control Median (Q1-Q3)	Social Isolation Median (Q1-Q3)
Body Weigt Changes	241.0 (239-247.25)	271.0 (265.75-275.75)*
FST		
Immobility time (sec)	12.87 (5.59-18.26)	87.46(42.39-246.41)*
Climbing time (sec)	59.41(38.89-73.18)	30.57 (27.16-35.69)*
Swimming time (sec)	219.59 (210.73-169.75)	189.22(68.66-234.24)*
OFT		
Crossed square	135.5(104.5-169.75)	91.0 (65.75-121.50)*
Time in center (sec)	25.0 (25.0-43.0)	11.0(5.0-15.5)*
Time in periphery (sec)	288.2(280.0-294.0)	274.5 (250.0-283.25)
Rearing	13.5 (6.75-15.5)	15.0 (10.0-19.0)
Grooming	1.5 (1.0-3.0)	2.0 (1.0-4.5)

*Note: *p < .05 vs. control.*

OFT Results

The median values of squares crossed, rearing, grooming frequency, time spent in the center, and time spent along the periphery are presented in Table 1. In the social isolation group, the number of squares crossed was significantly higher compared to the control group ($p < .05$). Time spent in the center was significantly reduced in the social isolation group relative to controls ($p < .05$), while time spent in the periphery was significantly increased ($p < .05$). No statistically significant differences were observed between the social isolation and control groups in grooming or rearing ($p > .05$).

FST results

The mean values of immobility, swimming, and climbing durations are presented in Table 1. Statistically significant differences were observed among all three parameters. In the social isolation group, immobility duration was significantly increased compared to the control group ($p < .05$). Swimming duration was significantly decreased in the social isolation group relative to controls ($p < .05$). Climbing duration was also significantly reduced in the social isolation group compared to the control group ($p < .05$).

Table 2.

MDA, GSH, TLR4, NF-κB, GSK-3β, and BDNF levels in the study groups. Results are presented as median (Q1–Q3) (n = 8).

Parameters	Control Median (Q1-Q3)	Social Isolation Median (Q1-Q3)
MDA (nmol/g tissue wet weight)	22.26 (20.59-22.26)	31.85 (26.45-41.10)*
GSH (µmol/g tissue wet weight)	5.46 (5.10-6.49)	5.11 (4.79-5.50)
TLR4 (pg/ mL)	1.80 (1.70-1.90)	1.95 (1.87-2.12)*
NF-κB (pg/ mL)	3.50 (3.30-3.87)	4.0 (3.70-4.42)*
GSK3β (pg/ mL)	7.60 (6.80-8.30)	9.10 (8.20-9.90)*
BDNF (pg/ mL)	1.0 (0.86-1.13)	0.90 (0.79-0.98)*

*Note: MDA: Malondialdehyde, GSH: reduced glutathione, TLR4: Toll like receptor 4, NF-κB λB: Nuclear factor κ B, GSK3β: Glycogen synthase kinase-3 beta, BDNF: Brain derived neurotrophic factor *p < .05 vs. control.*

Biochemical Analyses

Results PFC GSK-3β levels, shown in Table 2, were also significantly elevated in the social isolation group compared to controls ($p < .05$). In the social isolation group, PFC TLR-4 levels were significantly higher than those in the control group ($p < .05$). Similarly, PFC NF-κB levels were significantly increased in the social isolation group relative to the control group ($p < .05$). In contrast, no statistically significant differences were detected in prefrontal cortex BDNF levels between the groups. PFC MDA levels were significantly elevated in the social isolation group compared with controls ($p < .05$). However, no significant differences were observed in GSH levels between the groups.

Discussion

Our findings demonstrated that social isolation induced both depressive- and anxiety-like behaviors, accompanied by a significant upregulation of TLR4, NF-κB, and GSK-3β protein levels in the prefrontal cortex. These results align with previous evidence indicating that activation of the TLR4/NF-κB signaling cascade contributes to neuroinflammation and behavioral impairments in stress-related disorders (Jiang et al., 2024; Nie et al., 2018). Furthermore, the observed increase in GSK-3β expression supports its proposed role as a central regulator of neuroplasticity and mood-related processes (Dandekar et al., 2018). Collectively, these data suggest that the TLR4/NF-κB/GSK-3β axis may act as a convergent pathway linking neuroinflammatory signaling to the behavioral disturbances associated with chronic social isolation.

After six weeks of social isolation, rats displayed pronounced behavioral alterations, including prolonged immobility in the forced swim test (FST), a hallmark of depression-like behavior. Concurrently, open-field test (OFT) performance revealed diminished center exploration and enhanced thigmotaxis, supporting the presence of anxiety-like features. In line with numerous previous studies reporting isolation-induced behavioral abnormalities (Chmelova et al., 2019; Dávila-Hernández et al., 2021; Dimonte et al., 2023; Toth et al., 2011), SI-reared animals in our study exhibited pronounced anxiety- and depression-like behaviors in adulthood compared to controls. Taken together, these findings reinforce the notion that the social isolation paradigm induces enduring behavioral deficits that persist into adult life.

In the field of depression research, two prominent perspectives have attracted considerable attention: the inflammatory hypothesis and the neuroplasticity hypothesis, both of which are regarded as central frameworks for explaining the underlying pathophysiology (Du et al., 2024). BDNF levels have been strongly implicated in stress-related disorders, including depression and anxiety. Notably, social isolation has been shown to markedly reduce BDNF expression. For instance, a 14-day period of social isolation significantly decreased BDNF levels in rats (Aswar et al., 2022), other studies have reported no changes in hippocampal BDNF expression following 8 weeks of isolation (Chmelova et al., 2019). Our findings indicate that prolonged social isolation for six weeks had no significant effect on BDNF protein expression in the prefrontal cortex. While decreases in hippocampal BDNF mRNA and protein levels have been widely documented in socially isolated animals, relatively few studies have examined, or demonstrated, comparable changes in BDNF protein levels within the PFC (Murínová et al., 2017). Divergent results are likely attributable to differences in the duration of social isolation protocol and the strain of rats used.

SI rats also exhibited greater body weight compared to controls. Consistent with earlier reports, the type of stress appears to play a crucial role in regulating food consumption: whereas acute stress tends to suppress feeding behavior, chronic stress promotes it (Izadi et al., 2018). Accordingly, chronic isolation stress has been shown to result in persistent hyperphagia and binge-like eating behaviors (Dulabi et al., 2020). In the present study, post-weaning social isolation induced significant weight gain, which was accompanied by depressive- and anxiety-like behaviors.

Notably, protein expression levels of TLR3 and TLR4 have been reported to be significantly elevated in the prefrontal cortex of depressed suicide patients (Zhang et al., 2020). Furthermore, activation of the TLR4/MyD88/NF-κB signaling pathway has been shown to contribute to inflammatory responses in various depression models (Gárate et al., 2011; Shirayama et al., 2022). Increased TLR4 signaling has been suggested to interfere with neuroplasticity and neurotrophic support, which may contribute to depressive phenotypes (Shirayama et al., 2022). Consistent with this view, pharmacological suppression of TLR4 activity has been reported to exert antidepressant-like effects in male rodents (Dionisie et al., 2021). Consistent with these reports, our findings indicate that social isolation increased TLR4 levels, which in turn promoted lipid peroxidation and activated the NF-κB signaling pathway. The observed increase in NF-κB levels in the prefrontal cortex following six weeks of social isolation aligns with previous studies demonstrating NF-κB activation in the hippocampus and prefrontal cortex after chronic social isolation (Todorović and Filipović, 2017). One limitation of this study is the lack of direct measurement of pro-inflammatory cytokines in the social isolation model. Consequently, conclusions regarding

inflammation are limited to the assessed signaling pathways (TLR4 and NF-κB) and oxidative stress markers.

Oxidative stress induces the formation of free radicals that disrupt cellular components and overwhelm antioxidant defenses, ultimately leading to neuroinflammation and neuronal death (Abu-Elfotuh et al., 2022). Malondialdehyde (MDA), a byproduct of polyunsaturated fatty acid oxidation, has been reported to be elevated in depression. Moreover, oxidative stress and the excessive production of free radicals have been clinically recognized as key contributors to the development of depressive disorders (Chen et al., 2021). In the present study, following six weeks of social isolation, MDA levels in the prefrontal cortex were significantly elevated, whereas GSH levels remained unchanged. These findings are consistent with previous studies reporting that social isolation induces oxidative stress (Todorović and Filipović, 2017; Zhang et al., 2020). Collectively, our results support the growing body of evidence suggesting that oxidative stress constitutes a critical mechanism underlying the anxiety- and depression-like behaviors associated with social isolation.

Because GSH is the primary scavenger of free radicals in the brain, its depletion following chronic psychological stress, as well as in post-mortem PFC samples from patients with psychiatric disorders, underscores the importance of investigating the vulnerability of GSH-dependent defenses under stress conditions (Todorović & Filipović, 2017). Chronic stress has been reported to alter GSH levels, and several psychiatric disorders are characterized by GSH depletion (Todorović & Filipović, 2017). In response to oxidative stress, two GSH molecules are oxidized to glutathione disulfide (GSSG), which is subsequently reduced back to GSH by glutathione reductase (GR) (Giustarini et al., 2017). Previous studies have reported reduced GR activity following social isolation (Todorović & Filipović, 2017). In the present study, despite the absence of significant changes in GSH and MDA concentrations were significantly increased, Indicating enhanced lipid peroxidation. This pattern suggests that oxidative stress was initiated but had not progressed to a level sufficient to deplete intracellular glutathione stores. Given the dynamic regulation of glutathione metabolism, preserved GSH levels may reflect compensatory synthesis and /or efficient recycling mechanisms. Accordingly, the observed increased in MDA may represent an early or moderate oxidative insult occurring in the presence of maintained antioxidant capacity. Assessment of additional antioxidant enzymes may further clarify the extent of oxidative compensation and should be addressed in future studies.

In the present study, social isolation was found to increase GSK-3β activity, consistent with previous reports demonstrating elevated GSK-3β expression in the brains of socially isolated rats (Gong et al., 2017). The GSK-3β/β-catenin pathway has been strongly implicated in both the pathogenesis and treatment of depression (Xiao et al., 2021). Notably, increased GSK-3β activity has also been observed in the prefrontal cortex of individuals with depression who died by suicide (Gholami-Zanjanbar et al., 2024).

Conclusion

The present findings suggest that neuroinflammation induced by early-life social isolation may represent a critical mechanism linking stress to depression. Future studies are warranted to examine this relationship more comprehensively, particularly in the context of isolation-induced neurobehavioral impairments. Nevertheless, the absence of evaluations of sex differences and proinflammatory cytokine levels constitutes a limitation of the current study. Addressing these aspects in future investigations through more detailed analyses will be essential for clarifying the underlying mechanisms.

Ethics Committee Approval: Ethics committee approval was received for this study from the Local Ethics Committee of Zonguldak Bülent Ecevit University Animal Experiments (Protocol Date: September 12, 2019- Approval number: 2019-15-12/09).

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