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Hippocampal miR-34a/SIRT1 and BDNF Responses Following Adolescent Stress: Differential Effects of Ketamine and Fluoxetine

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ABSTRACT

Objective: Early life stress is a well-established risk factor for psychiatric disorders in adulthood; however, the underlying molecular mechanisms remain unclear. This study investigated the effects of chronic unpredictable mild stress (CUMS) starting in adolescence on anxiety- and depression-like behaviors, as well as on hippocampal miR-34a, SIRT1, BDNF, and NLRP3 levels in rats treated with ketamine or fluoxetine.

Methods: A total of 40 Wistar albino rats (4 weeks old) were divided into four groups: Control, CUMS, CUMS + fluoxetine (FLU), and CUMS + ketamine (KETA). Chronic unpredictable mild stress was applied for 6 weeks, while fluoxetine (10 mg/kg, i.p.) and ketamine (10 mg/kg, i.p.) were administered during the last 3 weeks. Behavioral assessments were performed at weeks 3 and 6 using the sucrose preference test (SPT), open field test (OFT), and forced swim test (FST). Hippocampal BDNF protein levels were measured by ELISA, and miR-34a, SIRT1, and NLRP3 gene expression levels were analyzed by RT-PCR.

Results: Both fluoxetine and ketamine improved CUMS-induced depression-like behaviors. Chronic unpredictable mild stress decreased hippocampal BDNF levels and increased miR-34a expression. Fluoxetine reversed the increase in miR-34a and elevated SIRT1 and BDNF levels. In contrast, although ketamine improved behavioral outcomes, it did not significantly alter hippocampal BDNF or SIRT1 levels and did not reduce miR-34a expression. No significant differences in NLRP3 expression were observed among the groups.

Conclusion: Adolescent CUMS induces depression-like behaviors by affecting the hippocampal miR-34a/SIRT1/BDNF axis. While the antidepressant effects of fluoxetine are associated with this axis, ketamine may exert its behavioral effects through different and possibly extra-hippocampal mechanisms. miR-34a may serve as a potential biomarker for depression related to early life stress.

Keywords: Depression, early life stress, fluoxetine, ketamine, miR-34a, SIRT1

INTRODUCTION

Early life is a critical period during which the neurodevelopmental process is not yet complete and sensitivity to environmental influences is high (Küçükkarapınar et al. 2021). Chronic stress experienced during this period is considered a significant risk factor in the etiology of many psychiatric disorders, such as depression, bipolar affective disorder, and obsessive-compulsive disorder in later life (McKay et al. 2022, Kilic et al. 2020). Furthermore, early life stress is reported to be associated with earlier onset of psychiatric disorders, a more unfavorable clinical course, inadequate response to treatment, and recurrent attacks (Tan et al. 2021).

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Significant changes occur in synaptic plasticity, myelination, and neurogenesis during adolescence. The incomplete maturation of the brain during this period makes the adolescent brain more sensitive to stress compared to the adult brain (Steinberg et al. 2006).

Clinical and preclinical studies are increasingly investigating neurocircuits, intracellular and extracellular mechanisms associated with chronic stress. However, the mechanisms by which antidepressant drugs can prevent neurobiological changes caused by chronic stress are also a significant area of research. Many studies have shown that chronic stress suppresses brain-derived neurotrophic factor (BDNF) synthesis in the hippocampus, that BDNF levels are low in depressed patients who have not used medication, and that BDNF levels increase with antidepressant treatment (Chen et al. 2001, Karege et al. 2002, Hacimusalar and Eşel 2018).

Sirtuins (SIRT), which are known to play a role in neuroplasticity similar to BDNF, play an important role in the pathophysiology of depression. It has been found that chronic stress causes a decrease in SIRT1, that pharmacological and genetic inhibition of SIRT1 function leads to depression-like behaviors, and that these changes are corrected by activating SIRT1 (Abe-Higuchi et al. 2016, Liu et al. 2016, Shen et al. 2018). In addition, the fact that SIRT1 affects oxidative stress response and the regulation of mitochondrial functions, as well as circadian gene expression, suggests that it may be linked to mood regulation (Uçak et al. 2025).

In recent years, microRNAs (miRNAs) have emerged as important regulators in terms of stress response, depression etiology, and antidepressant treatment response. In a large-scale study examining pharmacoepigenomic markers in schizophrenia, bipolar disorder, and major depressive disorder, miR-34a was identified as one of four miRNAs commonly found in all three disorders (Tsermpini et al. 2022). Preclinical studies have shown that miR-34a is expressed in the hippocampus, medial prefrontal cortex, basolateral amygdala, and dorsal raphe nucleus (Ye et al. 2022). It is thought that miR-34a plays a role in fundamental biological processes such as the cell cycle, apoptosis, and stress response; it exerts these effects by targeting certain mRNAs associated with synaptic plasticity, energy metabolism, and neural network activity (Iacono et al. 2020a, Ye et al. 2022). It has been suggested that exposure to chronic stress increases the expression of miR-34a, and that miR-34a exerts its effects by modulating the expression of BDNF and SIRT1 (Gao et al. 2010, Cui et al. 2017, Iacono et al. 2020a, Iacono et al. 2020b).

The NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasome is a multicomponent structure consisting of NLRP3, ASC, and caspase-1. Activation of the inflammasome leads to the maturation and release of the pro-inflammatory cytokines IL-1β and IL-18 via caspase-1. NLRP3 is known to be highly expressed, particularly in microglia, and plays an important role in the regulation of immune response, neuroinflammation, metabolic diseases, and neurological disorders. In subarachnoid hemorrhage, suppression of NLRP3 inflammasome activation via the SIRT1-mediated pathway has been reported to have a neuroprotective effect (Zhang et al. 2021). Furthermore, it has been shown that regulation of the SIRT1/NF-κB/NLRP3 signaling pathway can support microglial M2 polarization and synaptic plasticity, thereby partially ameliorating neuronal damage associated with ischemic stroke (Ou et al. 2023).

The pathophysiology of depression involves overactivation of the NLRP3 inflammasome, and NLRP3 has been reported to play a mediating role in the relationship between stress, pro-inflammatory cytokines, and depression (Iwata et al. 2013, Şahin and Arıcıoğlu 2014). Recent studies have demonstrated that the NLRP3 inflammasome is activated in depression and that the antidepressant effect occurs when this activation is inhibited (Pan et al. 2014, Zhang Y et al. 2015). In a previous study, we found that NLRP3 levels in the prefrontal cortex were increased in relation to early life stress in an experimental depression model, and this increase was reversed with vortioxetine treatment (Özdamar Ünal et al. 2024).

The delayed onset of response and insufficient benefit from treatment with current antidepressants, especially in cases of severe depression and high suicide risk, lead to difficulties in the clinical management of patients. The fact that approximately one-quarter of completed suicides are among those receiving antidepressant treatment (Karch et al. 2012), the high relapse rates of depression after remission (approximately 75%) (Gold et al. 2015), and the need for long-term maintenance treatment despite remission (Palazidou 2012) necessitate the investigation of new biological targets for the etiopathogenesis and treatment of depression. In this context, interest in the antidepressant effects of ketamine, an NMDA receptor antagonist, has increased in recent years due to its rapid onset of action and antisuicidal effects (Anzolin et al. 2024, Pastre et al. 2025). However, the mechanism mediating the antidepressant efficacy of chronic ketamine use has not been fully elucidated (Gibuła-Tarłowska et al. 2025).

There is substantial evidence supporting the relationship between stress and the hippocampus, a key component of the limbic system. The hippocampus is densely connected to brain regions known to be associated with stress, such as the prefrontal cortex and amygdala. Chronic and severe exposure to stress hormones can lead to suppression of neurogenesis and atrophy in the hippocampus. One study showed that adolescents who experienced early life stress had smaller hippocampal volumes and were more vulnerable to depression in adulthood (Rao et al. 2010).

Although the effects of chronic stress on molecules associated with neuroplasticity, epigenetic regulation, and neuroinflammation, such as BDNF, SIRT1, miR-34a, and NLRP3, have been described in adult animal models, adolescence represents a unique neurodevelopmental period. Therefore, the biological effects of stress and responses to antidepressant treatments may differ during this period. This study aimed to investigate the effects of chronic unpredictable mild stress on the hippocampal miR-34a/SIRT1/BDNF axis and the NLRP3 inflammasome during adolescence; and to comparatively determine which biological pathways fluoxetine and ketamine modulate stress-related behavioral and molecular changes.

METHODS

1. Experimental Animals: Wistar albino male rats (4 weeks old, 75±25 g, Süleyman Demirel University Experimental Animals and Medical Research Application and Research Center, Türkiye) were randomly divided into 4 groups of 10 rats each: 1) Control group; 2) chronic unpredictable mild stress (CUMS) group; 3) CUMS + fluoxetine (FLU) group; 4) CUMS + ketamine (KETA) group. After a 1-week adaptation period, the rats were housed in cages with 5 rats per cage. Unless otherwise specified, the experiments were carried out at a room temperature of 22±2 °C, 65–70% humidity, and a 12-hour light/dark cycle. Access to water and food was provided ad libitum unless otherwise specified. All stages of the experiment were approved by the Süleyman Demirel University Animal Experiments Local Ethics Committee with decision number 05/08–2020 dated 03.09.2020. All experiments were conducted in accordance with ARRIVE 2.0 (Animal Research: Reporting in Vivo Experiments) guidelines.

2. Chronic Unpredictable Mild Stress (CUMS) Model and Experimental Design: A recently reported meta-analysis in the literature indicated that the CUMS model was the most effective model among ten animal models of depression (Ratajczak et al. 2024). The CUMS was implemented as in our previous studies (Özdamar Ünal et al. 2022, Özdamar Ünal et al. 2024), adapted from the procedure suggested in the study by Burstein and Doron (2018). The experimental design is shown in Fig. 1.

Stressors consisted of 24 hours of water deprivation, 24 hours of food deprivation, 4 hours in a wet cage, 4 hours on moistened sawdust, 4 hours in a 45° tilted cage, 4 hours in an empty cage, 4 hours of social stress, 4 hours of physical restraint, and 24 hours of light exposure with disruption of the light-dark cycle. The timing of the stressors was standardized; the four-hour stressors were applied between 09:00 and 13:00, and the 24-hour stressors were always started at 09:00. An example of the stressors applied over one week is as follows: Day 1: 45° tilted cage (4 hours); Day 2: social stress (4 hours); Day 3: moistened sawdust (4 hours); Day 4: empty cage (4 hours); Day 5: physical restraint (4 hours); Day 6: disruption of the light-dark cycle (24 hours), and Day 7: water deprivation (24 hours). These stressors were repeated weekly for 6 weeks, with the order of stressors randomized.

3. Pharmacological Drug Administration: Ketamine (Interhas Medical and Chemical Products Industry and Trade Inc., Ankara, Türkiye) and fluoxetine (Lilly Pharmaceuticals Trade Ltd. Co., Istanbul, Türkiye) as a positive control were dissolved in sterile distilled water, and both were administered intraperitoneally at a dose of 10 mg/kg (Doboszewska et al. 2015, Michaëlsson et al. 2019). Considering the stressor effect of the injection, 0.9% sterile saline was administered intraperitoneally to the Control and CUMS groups. Fresh solutions were prepared immediately before administration and injected intraperitoneally at the same time every day (between 09:00 and 10:00) from days 22–42 of the experiment according to the experimental procedure.

4. Behavioral Tests: Behavioral tests were used to evaluate the success of the CUMS model and to assess the mental state of the rats. The tests were repeated three times: the first at the end of the adaptation period, and the others starting at day 21 and day 42 of the experiment, during the day (09:00–15:00) but in dim light (15 lux). They were administered at one-day intervals to avoid carry-over effects. The tests were administered in the order of open field test (OFT), forced swim test (FST), and sucrose preference test (SPT), progressing from non-invasive to invasive. All behavioral tests were performed by researchers unaware of which group the animals belonged to.

4.1. Sucrose Preference Test: The SPT is used to assess anhedonia, one of the two main symptoms of major depression. The test, originally developed by Willner et al. in 1987, was applied as in our previous study (Özdamar Ünal et al. 2022). The following formula was used to calculate the sucrose preference rate: sucrose preference rate (%)=sucrose consumption (g) / [water consumption (g) + sucrose consumption (g)] × 100.

4.2. Open Field Test: OFT is a test used to assess locomotor activity, exploratory behavior, and anxiety-related behaviors in rats (Doguc et al. 2021). The test was administered under the conditions described in our previous study (Özdamar Ünal et al. 2022). For the test, a black-painted wooden square box with a base divided into 16 equal squares of 25×25 cm with white lines, a wall height of 40 cm, and a floor of 100×100 cm was used. The test was recorded using a Sony SSC-DC398P top camera with the Smart Version 2.5 program.

The displayed floor is divided into three concentric imaginary quadrants: the center square, the inner quadrant, and the outer quadrant. Rats were individually released into the apparatus from a specific corner of the platform in a quiet

environment and allowed to explore freely for 5 minutes. During this time, the number of lines crossed with at least three extremities, rearing, walling, time spent in the center square, and the number of entries into the inner and center zones indicate locomotor activity. These are also evaluated as measures of exploration and anxiety. Increased instances of these behaviors are associated with increased locomotor activity, decreased anxiety, and increased exploration. Conversely, a longer time spent in the outer zone indicates decreased exploratory behavior and increased anxiety. An increase in the recorded number of urination and defecations is interpreted as favoring high anxiety (Mesembe et al. 2012).

4.3. Forced Swimming Test: The FST is a test used to assess behavioral despair, a predictor of depression in rats, and was applied as described in our previous study (Özdamar Ünal et al. 2022). Rats were individually placed in a transparent cylindrical tank 80 cm high and 20 cm in radius, containing 50 cm deep water at room temperature (23 °C). Their activity was recorded for 6 minutes using a top camera (Sony SSC-DC398P) with the Smart Version 2.5 program. Immobility was defined as the time the rat remained stationary and performed only the minimum movements necessary to stay afloat. Mobility was considered to be escape-oriented behaviors such as active swimming, jumping, rearing, sniffing, or diving. Increased immobility in rats during this test indicated behavioral despair and was associated with depressive mood (Slattery and Cryan 2012).

5. Tissue Dissection and Hippocampus Retrieval: Approximately 24 hours after the final behavioral test, rats were sacrificed under intraperitoneal ketamine (10%, 90 mg/kg) - xylazine (2%, 10 mg/kg) anesthesia. The rats were decapitated, and brain tissue and, from the brain tissue, hippocampal tissue were removed using specialized instruments. The hippocampal tissue obtained from one hemisphere was immediately transferred to an eppendorf tube containing 1 ml of cold phosphate buffer, while the hippocampus obtained from the other hemisphere was washed and placed in a 1.5 ml eppendorf tube free of RNase. The tissues were stored at -80 °C until analysis.

6. ELISA (Enzyme Linked Immunoabsorbent Assay) Analysis: Prior to analysis, hippocampal tissues from two animals within the same group were pooled to obtain sufficient protein amounts, and BDNF measurements were performed on five pooled samples per group (representing tissues from 10 animals). They were homogenized in a specially prepared homogenization buffer containing protease inhibitors (leupeptin, aprotinin, ethylenediaminetetraacetic acid-EDTA, and ethylene glycoltetraacetic acid-EGTA). Hippocampal tissue was analyzed using the ELISA method with a commercial BDNF E-Lab Science (Houston, Texas, USA) kit. The BDNF kit contains 7 standards with different concentrations ranging from 31.25 to 2000 pg/ml. Standards were pipetted in duplicate, and a standard curve was generated based on the obtained optical density values. The concentrations of the study samples were calculated using this graph. BDNF levels were reported as concentration per microgram of protein. The sensitivity limit of the kit for BDNF was reported as 18.75 pg/ml.

7. Gene Expression Analysis: Frozen hippocampal tissues were thawed, 1 ml of TriPure Isolation Reagent (Roche Diagnostics) was added, and the samples were homogenized. Total RNA was isolated using the High Pure RNA Tissue Kit (Roche Diagnostics). RNA concentrations were measured using spectrophotometric methods. The concentration and purity of the obtained RNA samples were determined by measuring their absorbance at 260 and 280 nm wavelengths. Complementary DNA (cDNA) synthesis was performed from the obtained total RNA samples using the RevertAid RT Reverse Transcription Kit (Thermo Scientific™) according to the manufacturer's instructions. Synthesized cDNAs were analyzed using primers designed for the specified gene regions on a Roche LightCycler 480 II instrument with a LightCycler® 480 SYBR Green I Master. The sequences of the PCR primers for Sirtuin-1 (NM_001372090), NLRP3 (NM_001191642.1), and ACTB (NM_031144.3) are given in Table 1. The ACTB (β-actin) gene was used as an internal control (housekeeping gene). The threshold cycle (CT) of the investigated genes was determined, and normalized according to ACTB the housekeeping gene. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the data were compared to the control group.

8. miRNA Expression Analysis: cDNA synthesis was performed from the obtained total RNA samples using a universal cDNA synthesis kit (Exiqon, Qiagen miRCURY LNA RT Kit) according to the manufacturer's instructions. Using synthesized cDNA as a template, RT-qPCR analysis of selected miRNAs with specific LNA primers and SYBR Green dye was performed using a Roche LightCycler 480 II instrument. The RNU6B gene was used as an internal control (housekeeping gene). The Ct of the investigated genes was determined and normalized with respect to the RNU6B housekeeping gene. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the data were compared to the control group.

Table 1. Sequences of PCR primers

GENES	
Sirtuin-1	5'-CCTCCTCATTGTTATTGGGTCT-3' (sense)
	5'-GAGGCAGAGGTTCCCTATTTATT-3' (antisense)
NLRP3	5'-TTTCCCAGACCCTCATGTTG-3' (sense)
	5'-GGTGCTGAGACTTGAGAAGAG-3' (antisense)
ACTB	5'-ACCACCATGTACCCAGGCATT-3' (sense)
	5'-CCACACAGAGTACTTGCGCTCA-3' (antisense)

9. Statistical Analysis: Statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS) program version 22 software. A p value of <0.05 was considered statistically significant. Behavioral test data were obtained by repeating the tests on day 1, day 21, and day 42. The data were evaluated using repeated measures analysis of variance in a factorial design. The validity of Mauchly's sphericity assumption was assessed. Following the analysis of variance, the Tukey test was used as a multiple comparison analysis. The Box M test was used to check the homogeneity of the variance-covariance matrix, and the Shapiro-Wilk test was used for the normality test. ANOVA was used to compare groups in the evaluation of BDNF levels. The Bonferroni test was used as a post-hoc test. Differences in NLRP3 and SIRT1 gene expression and miR-34a expression were given as fold changes using the $2^{-\Delta\Delta Ct}$ value. ANOVA was used to show the differences between groups in the statistical analysis of these data. The Dunnett test was used as a post-hoc test. Correlation analysis of non-parametric variables was performed using Spearman correlation. Multivariate analysis of covariance (MANCOVA) was applied to evaluate the relationship between behavioral changes and molecular changes. In the analysis, behavioral parameters were included as dependent variables, experimental groups as fixed factors, and molecular markers (BDNF, miR-34a, SIRT1, and NLRP3) as covariates.

The sample size was determined a priori using G*Power based on one-way ANOVA (four groups). A large effect size (Cohen's f = 0.45) was selected based on previous CUMS studies reporting behavioral and molecular alterations (Willner et al. 1987, Bai et al. 2012). With α = 0.05 and 80% power, the required sample size was estimated as 10 animals per group.

RESULTS

Early life stress (ELS) is associated with depression-like behaviors in adulthood. This study showed that ketamine and fluoxetine treatments improved depression-like behaviors associated with ELS.

When each group was evaluated within the group in the SPT, no significant change was observed over time in the control group. However, in the groups exposed to chronic stress, sucrose preference decreased significantly compared to baseline on day 21 (p <0.05). In the CUMS+FLU and CUMS+KETA groups, this difference disappeared on day 42 (p >0.05). Based on these findings, it can be concluded that fluoxetine and ketamine treatments improve anhedonia-like behavior induced by CUMS.

When intergroup data were compared for each measurement; while there was no significant difference between groups at baseline (p >0.05), sucrose preference rates were found to be lower in all stress groups compared to the control group on day 21 (p <0.05). In the CUMS group, the sucrose preference rate was significantly lower on day 42 compared to the control group (p <0.05), while the CUMS+KETA and CUMS+FLU groups were found to be not significantly different from the control group (p >0.05) (Fig. 2).

Mauchly's sphericity assumption was met in the evaluation of mobility duration data in FST (0.907; df=2, p=0.180). There was a significant effect of time (p <0.0001, F=122.20, df=2) and time-group interaction (p <0.0001, F=5.357, df=6). When each group was evaluated according to days, no significant difference was found in terms of mobility duration data of the control group on days 1, 21, and 42. In the stress group, the duration of mobility was significantly lower on days 21 and 42 compared to baseline (p <0.05). Both the CUMS+KETA and CUMS+FLU groups showed a significant decrease in mobility duration on day 21 compared to baseline (p <0.05), while mobility duration increased on day 42 after treatment (Figure 3).

Mauchly's sphericity assumption also applies to the immobility duration data (0.844; df=2, p=0.051). There is a daily variation (p <0.0001, F=83.096, df=2) and a time-group interaction (p <0.0001, F=5.707, df=6). Immobility duration is the portion of the total 6 minutes (360 seconds) recorded that is not included in the mobility duration. Therefore, the changes observed in mobility duration were detected in the same groups with the same measurements, as indicated in Fig. 3. While a decrease in mobility duration and an increase in immobility duration were observed on day 21 in all stress-treated groups compared to the control group (p <0.05), this decrease reversed on day 42 in the groups treated with fluoxetine and ketamine along with stress (p <0.05). These findings suggest that fluoxetine and ketamine improve immobility caused by chronic stress (Fig. 3).

In the OFT, no significant difference was found in the measurements taken, either between weeks or between groups (p >0.05). In the OFT, the number of lines crossed by rats within a certain period, the time spent in the outer zone, the time spent in the inner zone, the distance traveled in the center square, the total distance traveled, the number of entries into the inner quadrant, and the number of entries into the center were statistically analyzed (Fig. 4). In addition, the number of climbing (walling), rearing, urination, and defecation events were also analyzed.

The difference between the groups in terms of BDNF protein concentration levels measured in the rat hippocampus was found to be statistically significant (df=3, F=3.86, p=0.03). Although BDNF levels were found to be lower in the CUMS group, the difference between CUMS and the CUMS+FLU group was found to be statistically significant (p=0.043) (Fig. 5). These findings may indicate that CUMS reduces BDNF and that this change is reversed with fluoxetine treatment.

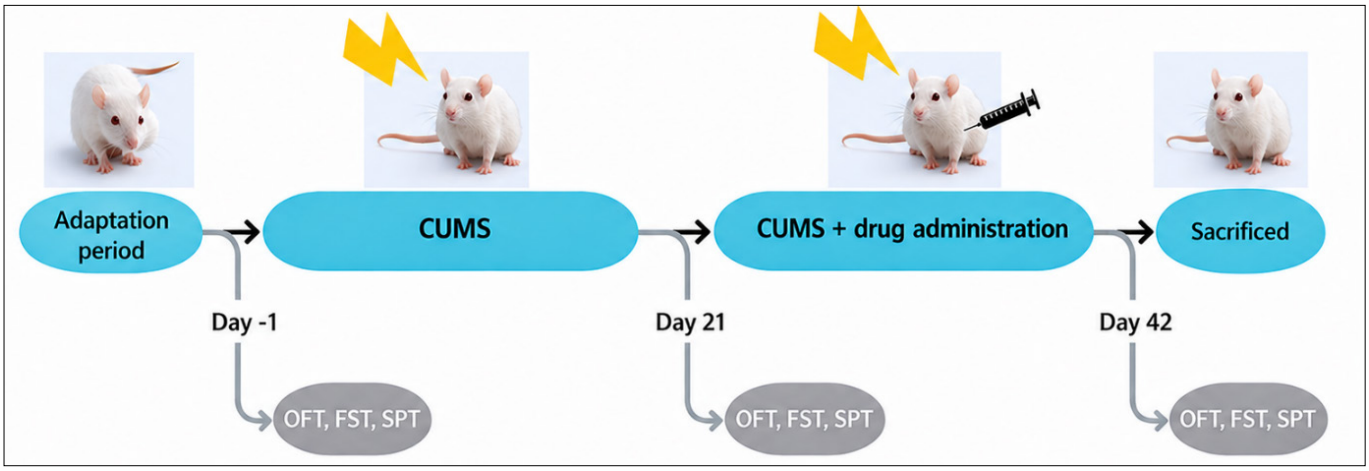

Figure 1. Schematic diagram of the experimental design. Wistar albino male rats (4 weeks old) were randomly assigned to four groups: Control (n=10), CUMS (n=10), CUMS+KETA (n=10), CUMS+FLU (n=10). The CUMS, CUMS+KETA, and CUMS+FLU groups were exposed to variable stressors at different times of the day for 42 days. Saline was administered to the Control and CUMS groups, ketamine to the CUMS+KETA group, and fluoxetine to the CUMS+FLU group daily for the last 21 days of the experiment. OFT, FST, and SPT were administered three times at one-day intervals: at the end of the adaptation period, after 21 days of CUMS administration, and after 42 days of CUMS administration (21 days of drug administration), respectively. CUMS represented chronic unpredictable mild stress; OFT represented the open field test; FST represented the forced swim test; SPT represented the sucrose preference test; D-1, day 1; D-21, day 21; D-42, day 42.

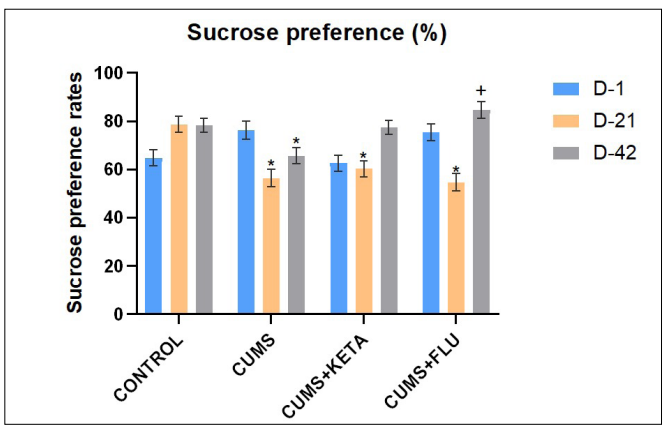

Figure 2. Results of repeated sucrose preference test measurements by groups according to days (D-1, D-21, D-42). Values are taken as mean ± SEM for each group. *: indicates a significant difference from the control group. +: indicates a significant difference from the CUMS group (p <0.05). CUMS: chronic unpredictable mild stress; KETA: ketamine; FLU: fluoxetine.

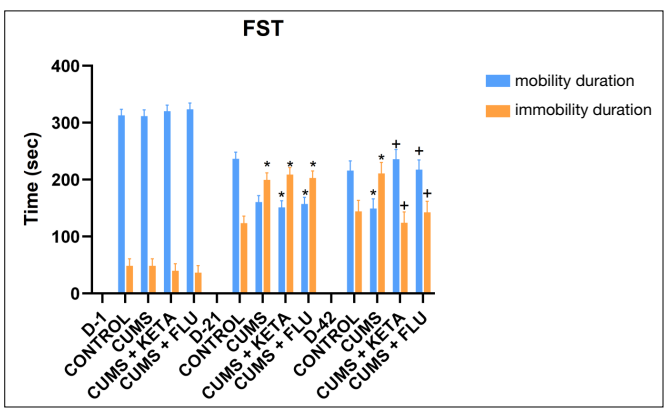

Figure 3. Within-group and between-group comparison of mean values of mobility and immobility durations of groups in the forced swimming test, according to days (D-1, D-21, D-42). Values are taken as mean ± SEM for each group. *: indicates a significant difference from the control group, +: indicates a significant difference from the CUMS group (p <0.05). CUMS: chronic unpredictable mild stress; KETA: ketamine; FLU: fluoxetine; FST: forced swimming test.

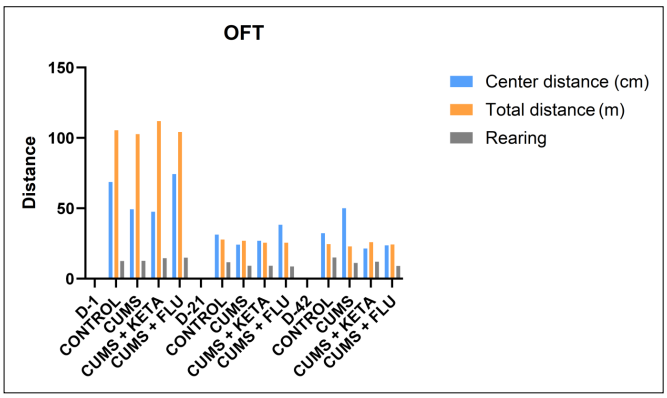

Figure 4. Repeated open-field test (OFT) measurement results for groups by day (D-1, D-21, D-42). Values are given as mean ± SEM for each group. A p-value <0.05 indicates a statistically significant difference. CUMS: chronic unpredictable mild stress; KETA: ketamine; FLU: fluoxetine; OFT: open-field test.

Furthermore, it can be concluded that chronic ketamine improves depression-like behaviors associated with ELS but does not have a significant effect on hippocampal BDNF.

The effects of ELS, fluoxetine, and ketamine treatments on NLRP3, SIRT1, and miR-34a expression levels in the rat hippocampus were investigated. Changes in SIRT1 gene expression were found to be statistically significant between the control and other groups (df=3, F=3.886, p=0.018). A statistically significant increase was observed in the CUMS+FLU group compared to the control group (FC=1.97, p=0.004). The changes in the CUMS and CUMS+KETA groups were not statistically significant (FC=0.40, p=0.530; FC=0.49, p=0.094, respectively) (Fig. 6).

Changes in miR-34a expression were found to be statistically significant between the control and other groups (p=0.004). Compared to the control group, the miR-34a expression levels were significantly higher in the CUMS and CUMS+KETA

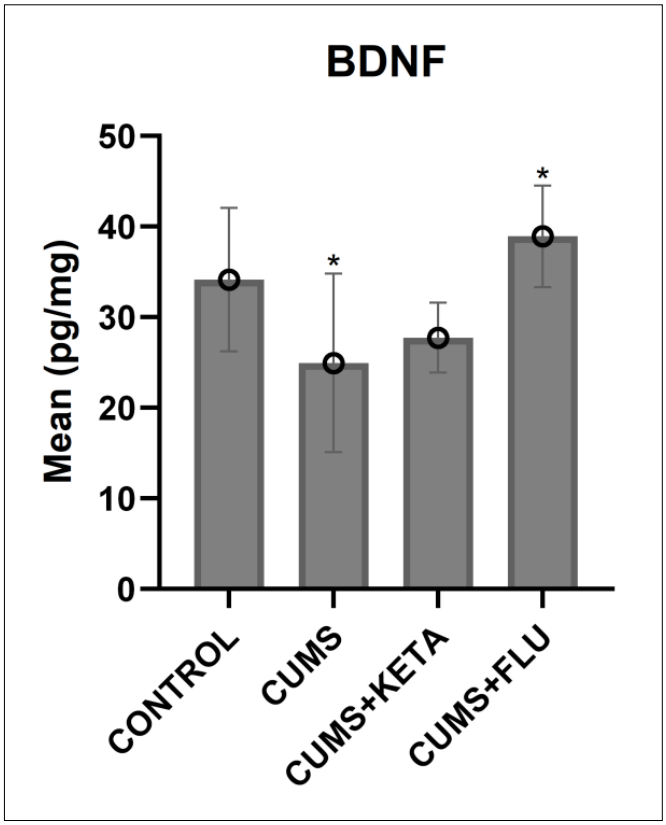

Figure 5. BDNF protein concentrations in HPC. Data are presented as mean ± standard deviation. * indicates a statistically significant difference (p <0.05). BDNF: Brain-derived neurotrophic factor; CUMS: chronic unpredictable mild stress; KETA: ketamine; FLU: fluoxetine.

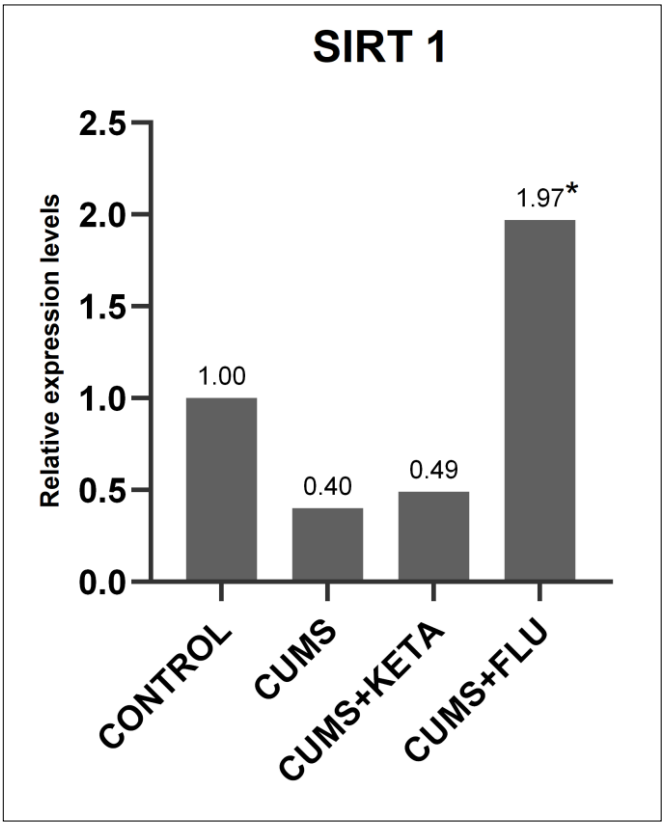

Figure 6. Hippocampal SIRT 1 gene expression by group. Data are presented as normalized mRNA expression in $\log_2(2^{-\Delta\Delta Ct})$. *: indicates a statistically significant difference compared to the control group (p<0.05). CUMS: chronic unpredictable mild stress; KETA: ketamine; FLU: fluoxetine.

groups (FC=1.94, p=0.004; FC=1.36, p=0.047, respectively). Compared to the CUMS+FLU group, it was found to be significantly higher in the CUMS and CUMS+KETA groups (p=0.003; p=0.039, respectively). Compared to the CUMS group, it was found to be significantly lower in the control and CUMS+FLU groups (p=0.015 and p=0.013, respectively) (Fig. 7).

NLRP3 gene expression did not differ significantly among the groups (p=0.416). Compared with the control group, no significant changes in expression levels were observed in the CUMS (FC = 1.59, p=0.245), CUMS+FLU (FC = 1.41, p=0.555), or CUMS+KETA groups (FC = 0.99, p=0.882). (Fig. 8).

The correlations between hippocampal NLRP3, SIRT1, miR-34a expression levels and BDNF protein concentrations were examined using Spearman correlation analysis. A significant positive correlation was found between NLRP3 and SIRT1 (r=0.426, p=0.009), while no significant correlation was found between NLRP3 and miR-34a, BDNF (r=0.068, p=0.691; r=-0.044, p=0.855, respectively).

MANCOVA results showed that the group factor had a significant effect on the combination of behavioral parameters [Pillai's Trace=1.212; F (18,78)=2.937; p=0.001]. This finding reveals that the applied chronic stress model

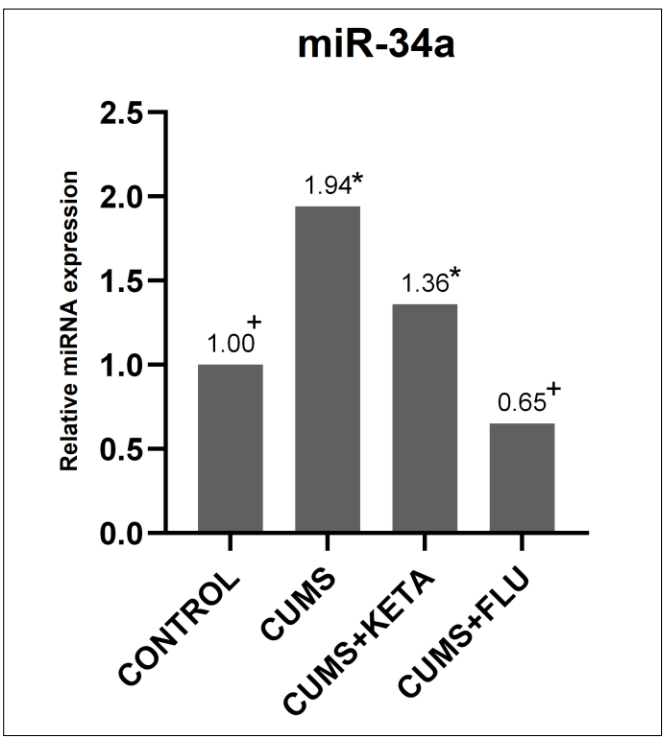

Figure 7. Hippocampal miR-34a expression by group. Data are presented as $\log_2$ $(2^{-\Delta\Delta Ct})$ normalized mRNA expression. *: Indicates a statistically significant difference compared to the control group; +: Indicates a statistically significant difference compared to the CUMS group (p<0.05). CUMS: chronic unpredictable mild stress; KETA: ketamine; FLU: fluoxetine.

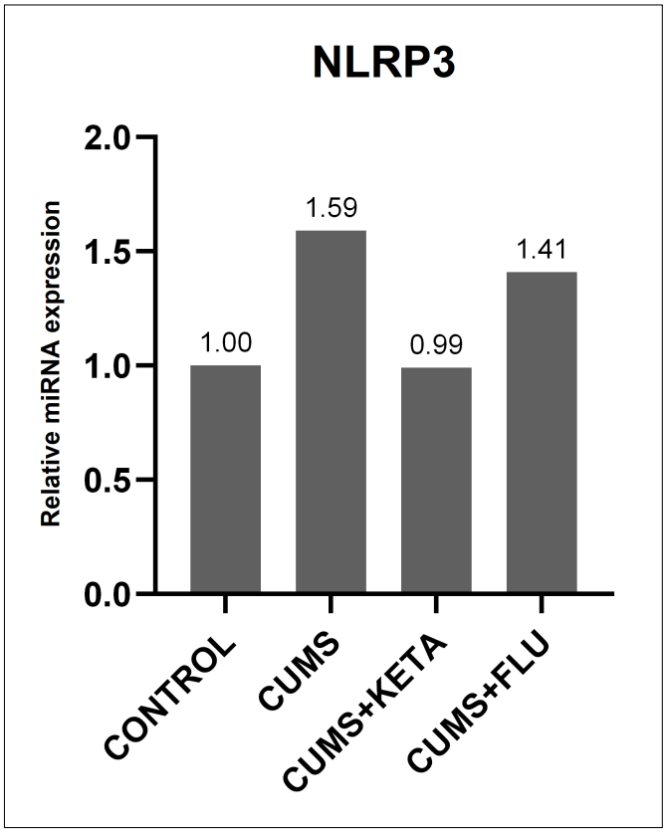

Figure 8. Hippocampal NLRP3 gene expression by group. Data are presented as normalized mRNA expression in $\log_2(2^{-\Delta\Delta Ct})$. No statistically significant differences were found between the groups (p>0.05). CUMS: chronic unpredictable mild stress; KETA:ketamine; FLU: fluoxetine.

leads to significant differences in behaviors. When molecular variables were examined, it was found that SIRT1 levels were significantly related to behavioral parameters [Pillai's Trace=0.413; F (6,24)=2.819; p=0.032]. In contrast, BDNF (p=0.797), miR-34a (p=0.704), and NLRP3 (p=0.968) variables did not have a significant multivariate relationship with behavioral measures.

In univariate follow-up analyses, the group effect was particularly prominent in FST. Significant group differences were found in the mobility duration [F (3,29)=9.878; p <0.001] and immobility duration [F (3,29)=10.277; p <0.001] variables measured after 3 weeks of CUMS. In addition, immobility duration measured after the completion of 6 weeks of CUMS and 3 weeks of treatment applications was also significantly affected by the group factor [F (3,29)=3.943; p=0.018]. These findings suggest that chronic stress and treatment processes lead to significant and measurable differences in behavior.

DISCUSSION

Early life is a developmental stage with high vulnerability to psychopathology, during which stress response systems and neuroplastic processes are reshaped. This study shows that CUMS beginning in adolescence leads to depression-like behaviors in adulthood and leads to significant changes in neuroplasticity, epigenetic regulation, and biological pathways associated with stress response in the hippocampus. Our findings reveal that both fluoxetine and ketamine reduce anhedonia and behavioral despair-like symptoms, but the underlying biological mechanisms of this improvement differ. In this respect, the study indicates that behavioral improvement in the treatment of depression due to early life stress may not occur solely through a common molecular pathway.

In this study, anhedonia and behavioral despair, one of the main behavioral components of depression, were evaluated with the SPT and FST. The SPT results showed that CUMS initiated in adolescence leads to significant anhedonia-like behavior in adulthood. This is characterized by a significantly decreased preference for sucrose compared to the control group. Remarkably, the disappearance of the observed decrease in sucrose preference rates in the fluoxetine and ketamine-treated groups suggests that both treatments effectively improve anhedonia-like behavior induced by chronic stress. The literature also reports that chronic stress patterns lead to a significant decrease in sucrose preference, which is a reliable preclinical indicator of anhedonia and is reversible with classic antidepressant treatments such as fluoxetine (Grippo et al. 2006). Furthermore, it has been shown that rapidly acting agents such as ketamine reverse stress-induced anhedonia and bring sucrose preference closer to normal (Kingir et al. 2023).

Similarly, FST findings suggest that chronic stress leads to behavioral despair-like changes consistent with decreased activity time and increased immobility duration. In groups treated with fluoxetine and ketamine in conjunction with stress, the increase in mobility duration and decrease in immobility duration on day 42 demonstrates that both drugs can reverse stress-induced behavioral despair. These findings are consistent with studies in the literature reporting that stress reduces mobility and increases immobility in FST, and that these changes can be corrected with antidepressant treatments (Réus et al. 2015, Fitzgerald et al. 2019).

In this study, no significant anxiety-like behaviors were detected in the OFT using the CUMS model. A recently published meta-analysis revealed that OFT results show a high degree of variability, and that interstudy differences are strongly influenced by several methodological and biological factors such as age, gender, test environment, habituation process, animal care, and the selection of measured parameters (Çevik et al. 2025). It has also been noted that repeated application of the test may dampen stress responses and lead to adaptation, making it difficult to distinguish differences between control and stress groups (Bosch et al. 2022). The type and duration of stress also significantly affect behavioral responses, and chronic stress has been reported to be associated with depression-like behaviors rather than

increased anxiety (Simões et al. 2025). In addition, the higher exploratory behavior and more variable anxiety responses shown by adolescent rats (Sturman and Moghaddam 2011) may limit the sensitivity of OFT in revealing anxiety-like behaviors in this age group. For these reasons, the lack of a significant difference in OFT in our study is consistent with both the variable effects of the CUMS model on anxiety and the methodological limitations of the test.

This study showed that early life stress leads to a decrease in BDNF levels in the hippocampus. It has been previously reported that this decrease due to early life stress may be permanent and may form the biological basis of susceptibility to depression in adulthood. Indeed, a study comparing the effects of the CUMS model in adulthood and the maternal deprivation paradigm with early life stress showed that only stress applied in early life led to decreased BDNF levels in the hippocampus (Bai et al. 2012). These findings suggest that early BDNF changes may be associated with depression-like behaviors that emerge in adulthood. Consistent with our findings, previous studies have also shown that chronic administration of fluoxetine increases hippocampal BDNF levels (Hodes et al. 2010). In contrast, in this study, chronic ketamine treatment did not produce a significant change in hippocampal BDNF levels, although it did lead to behavioral improvement. It has been reported that chronic ketamine administration at doses of 5, 10, and 15 mg/kg has limited effects on hippocampal BDNF levels, whereas acute, high-dose administration can induce transient increases (Garcia et al. 2008a, 2008b). This suggests that the rapid antidepressant effects of ketamine may not be sustained long-term via hippocampal BDNF.

One of the candidate regulators that can shape the observed changes in BDNF at the epigenetic level is miR-34a. It has been reported that miR-34a expression in the hippocampus of adult rats is increased with exposure to chronic stress and decreased with fluoxetine treatment (Yi et al. 2020). Similarly, it has been shown that serum miR-34a-5p expression is increased in patients with depression and decreased with paroxetine treatment (Kuang et al. 2018). In this study, it was also found that miR-34a increased with chronic stress and this difference disappeared with fluoxetine treatment. In contrast, chronic ketamine treatment was found to have no significant effect on miR-34a expression. Previous studies have reported that increased miR-34a affects BDNF and SIRT1 expression (Cui et al. 2017, Kuang et al. 2018, Xu et al. 2020) and that miR-34a/SIRT1 levels can be associated with major depression symptoms, especially in cases with early life stress (Iacono et al. 2020b). It has been shown that miR-34a inhibits SIRT1 expression by binding to its 3′UTR region (Yamakuchi et al. 2008, Fu et al. 2017), and the stabilizing effect of fluoxetine on the miR-34a/SIRT1 axis was also observed in this study.

Preclinical studies have reported that ketamine can increase miR-34a expression in hippocampal tissue and lead to significant apoptosis, particularly in CA1 neurons (Jiang et al. 2014, Ye et al. 2022). Conversely, it has been suggested that ketamine exerts antidepressant effects in the short term by increasing neuroplasticity, strengthening synaptic connections, and modulating neuroinflammation in the medial prefrontal cortex; however, it may exhibit different and region-specific effects at the molecular level in the long term (Gass et al. 2019, Shinohara et al. 2021). Considering these findings together, it is thought that the effects of ketamine on miR-34a may occur in different brain regions, such as the prefrontal cortex, rather than the hippocampus, and through time-sensitive mechanisms.

It has been previously shown that chronic stress in adulthood reduces SIRT1 activity in the hippocampus and that SIRT1 activation leads to antidepressant-like effects (Abe-Higuchi et al. 2016, Liu et al. 2016). However, in this study, no significant difference was found in SIRT1 mRNA expression with exposure to chronic stress. Similarly, it has been reported that SIRT1 protein expression does not change in the CA1, CA3, and dentate gyrus regions of the hippocampus after chronic stress, but activity can increase in CA3 and the dentate gyrus (Ferland and Schrader 2011). Therefore, it is thought that the SIRT1 response may differ in hippocampal subregions and that analyzing the entire hippocampus as a single sample may mask regional variations. However, it should be considered that the mRNA level of SIRT1 was measured in this study, and the mRNA-protein correlation is not always strong, and the functional deacetylase activity of SIRT1 may vary independently of protein amount. Indeed, the literature reports that the results regarding SIRT1 under chronic stress conditions can vary depending on the model, duration, and measurement level (Qiu et al. 2023).

In this study, the detection of an increase in hippocampal SIRT1 levels with fluoxetine treatment is consistent with the literature reporting the effects of SSRIs on hippocampal plasticity and epigenetic regulatory mechanisms in chronic application (Li et al. 2020). The fact that ketamine treatment did not cause a significant change in hippocampal SIRT1 levels suggests that the antidepressant effects of ketamine occur through brain region-specific mechanisms. A recent study showed that the antidepressant effect of S-ketamine was associated with SIRT1-mediated BDNF increase in the medial prefrontal cortex (Hou et al. 2022). These findings indicate that the effect of ketamine on SIRT1 may occur in circuits associated with rapid synaptic plasticity, such as the medial prefrontal cortex, rather than the hippocampus. It is also possible that the hippocampal effects of ketamine were not detected in the current experimental timing due to their time-sensitive and transient nature.

In this study, the expression of NLRP3, whose main function is to regulate the pro-inflammatory cytokines IL-1β and IL-18 by activating caspase-1, was examined in the hippocampus, and no significant difference was found between the groups (Pan et al. 2014). However, in our previous study, it was shown that prenatal maternal stress increased NLRP3 activation in both the mother's and offspring's hippocampus (Özdamar Ünal et al. 2022). This difference may be related to the timing and duration of stress application, as well as the variability of NLRP3 response among brain regions. Indeed, it has been reported that chronic stress increases IL-1β-related inflammation in the prefrontal cortex, and this is linked to microglial NLRP3 inflammasome activation (Pan et al. 2014, Zhang GF et al. 2015). The reduction of this activation with fluoxetine administration (Pan et al. 2014, Wang et al. 2020) suggests that the antidepressant effect may occur through microglial NLRP3 regulation in the prefrontal cortex rather than the hippocampus.

It is suggested that anti-inflammatory processes are involved in the antidepressant mechanism of action of chronic ketamine treatment, and that NLRP3 may mediate this effect. Early life stress increases pro-inflammatory cytokines in serum and cerebrospinal fluid; it has been reported that ketamine can reverse these effects, generating an anti-inflammatory response and suppressing the NLRP3 inflammasome (Réus et al. 2015, Li et al. 2019). However, the limited findings regarding ketamine's effect on NLRP3 and inflammatory processes, the lack of clarity regarding dose-duration-brain region specificity, and the possibility of more pronounced effects in non-hippocampal regions should be considered in interpreting our findings.

In this study, the positive correlation found between hippocampal NLRP3 and SIRT1 levels while seemingly contradictory to the suppressive role of SIRT1 on NLRP3 reported in the literature (Li et al. 2016), is thought to be explainable by compensatory regulatory responses developing under inflammatory load. It has been reported that chronic inflammation and oxidative stress trigger NLRP3 inflammasome activation, while cellular stress response and epigenetic regulators such as SIRT1 act as compensatory mechanisms to limit inflammation (Kauppinen et al. 2016). Furthermore, considering that the parameters measured in this study reflect expression levels and that the functional activity of SIRT1 was not evaluated, it should be considered that increased SIRT1 expression may not necessarily mean that inflammatory suppression has occurred.

In this study, the relationship between behavioral changes and molecular changes was evaluated using a multivariate statistical approach, not limited to group comparisons alone. MANCOVA results confirmed that the experimental groups had significant effects on behavioral outcomes, while also showing that some molecular variables may be predictive of these behavioral changes. In particular, the significant relationship between SIRT1 and behavioral parameters suggests that this molecule may play an important role in the regulation of stress-related behaviors. Given SIRT1's known regulatory effects on neuroplasticity, mitochondrial function, and stress response, these findings suggest that SIRT1 might be a potential mediator between molecular-level changes and behavioral phenotypes. In contrast, the lack of a significant multivariate association between BDNF, miR-34a, and NLRP3 variables and behavioral measurements suggests that the effects of these markers may arise through context-specific or nonlinear mechanisms. This necessitates a more detailed and diverse experimental approach to evaluating the effects of these molecular markers on behavioral outcomes. The multivariate statistical approach used in this study demonstrates that behavioral changes are not merely a result of group effects, but that specific molecular changes, particularly SIRT1, make significant contributions to these behavioral outcomes. In this respect, the study reveals a holistic relationship between behavioral and molecular findings, enhancing the power to interpret the mechanisms of action of chronic stress and drugs.

This study has some methodological limitations. Firstly, the experiments were conducted only on male rats, limiting the generalizability of the findings to female animals. Given the significant role of sex differences in stress response, neuroplasticity, and response to antidepressant treatment, future studies should evaluate both sexes. Secondly, molecular analyses were limited to hippocampal tissue; brain regions associated with stress response, emotion regulation, and especially the antidepressant effect of ketamine, such as the prefrontal cortex and amygdala, were not examined. This may have contributed to the incomplete elucidation of ketamine's region-specific and time-sensitive effects. Furthermore, the lack of complete correlation between the findings in this study and adult CUMS models may be related to neurodevelopmental characteristics specific to adolescence. Adolescence is a critical developmental period in which neuroplasticity, epigenetic regulation, and stress response systems are restructured, and stress exposure during this period may have different and more lasting molecular effects compared to adults. It is known that early life stress can create significant effects (Steinberg et al. 2006, Sturman and Moghaddam 2011). Indeed, it has been reported that early life stress can affect immune responses and miRNA-BDNF regulation for a longer period than in adult models (Bai et al. 2012, Tan et al. 2021). In this context, although ketamine has been reported to have an antidepressant effect via hippocampal BDNF and SIRT1 in adult studies (Abe-Higuchi et al. 2016, Hou et al. 2022), it is possible that the behavioral improvement observed in our study occurred through different brain regions or specific neural circuits

rather than the hippocampus. Furthermore, molecular markers were primarily evaluated at the expression level in this study; protein activity, post-translational regulations, and functional outcomes were not analyzed. Finally, the limited sample size may have contributed to the statistical inability to determine some biological effects. Despite these limitations, the study's holistic approach to the behavioral and biological consequences of chronic stress initiated in adolescence in adulthood makes a significant contribution to the literature.

CONCLUSION

This study demonstrates that chronic, unpredictable, mild stress beginning in adolescence leads to symptoms such as anhedonia and behavioral despair in adulthood, and that these behavioral changes are accompanied by alterations in neuroplasticity, epigenetic regulation, and stress response-related biological pathways in the hippocampus. Our findings suggest that fluoxetine exhibits a more pronounced and holistic biological effect via the miR-34a/SIRT1/BDNF axis, whereas ketamine, despite its behavioral efficacy, differs through classical hippocampus-centered neuroplasticity markers and likely acts through region- and time-specific mechanisms. These results reveal that the biological basis of depression due to early life stress cannot be evaluated independently of the developmental context, and that neurodevelopmental processes specific to adolescence may shape the mechanisms of antidepressant action. It is believed that this holistic approach, which takes into account the timing of early life stress and the developmental state of the brain, may contribute to the development of more targeted and individualized preventive and therapeutic strategies in the future.

Ethics Committe Approval: The study protocol was approved by the Süleyman Demirel University Animal Experiments Local Ethics Committee on 03.09.2020, with decision number 05/08–2020.

Conflict of Interest: The authors declare no conflicts of interest.

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