RESEARCH PAPER

# Neurobehavioral and Gene Expression Modulation by L-Theanine in a Chronic Unpredictable Mild Stress Model in Wistar Albino Rats

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**Abstract**

**Background:**

Chronic stress contributes significantly to anxiety and depression through dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis and associated molecular pathways. L-theanine, a natural amino acid, exhibits potential neuroprotective and anxiolytic effects.

**Objective:**

To evaluate the effects of L-theanine on behavioral and gene expression alterations in an Unpredictable Chronic Mild Stress (UCMS)-induced Wistar albino rat model.

**Methods:**

Wistar albino rats were subjected to UCMS to induce chronic stress. Behavioral assessments were performed using the Elevated Plus Maze (EPM) and actophotometer to evaluate anxiety-like behavior and locomotor activity, respectively. Gene expression analysis was conducted using RT-PCR for key stress-related genes including **BDNF, GABRA1, CRHR2, FADD, CDK6,** and **NR3C1**.

**Results:**

UCMS significantly induced anxiety-like behavior, evidenced by reduced time spent in open arms and increased time in closed arms in the EPM, along with decreased locomotor activity. L-theanine treatment significantly improved these behavioral parameters compared to the stress group and showed comparable or superior effects to fluoxetine. At the molecular level, UCMS caused downregulation of **BDNF, CRHR2, CDK6,** and **NR3C1**, and upregulation of **GABRA1** and **FADD**. L-theanine treatment effectively restored these gene expression changes toward normal levels.

**Conclusion:**

L-theanine exhibits significant anxiolytic and antidepressant-like effects by improving behavioral outcomes and modulating stress-induced gene expression. These findings highlight its therapeutic potential in managing stress-related neuropsychiatric disorders.

**Keywords:**

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## Introduction

Animal models play a crucial role in understanding the neurobiological basis of behavioral and psychiatric disorders. Rodents, particularly Wistar albino rats, are widely used in behavioral neuroscience due to their well-characterized physiology and structural similarities to the human brain (Von Borell, E.H., 2001). These models allow researchers to simulate human-like emotional and cognitive responses under controlled experimental conditions, thereby facilitating the study of stress-related disorders and potential therapeutic interventions (Cooper et al., 2007).

Chronic stress is a major contributing factor in the pathophysiology of anxiety and depression. The **Unpredictable Chronic Mild Stress (UCMS)** model is a well-established experimental paradigm that mimics human depression by exposing animals to a series of mild, unpredictable stressors over time. This leads to behavioral, neurochemical, and molecular alterations resembling depressive states. Behavioral assessment in such models is commonly performed using validated tools such as the **Elevated Plus Maze (EPM)** and **Actophotometer**. The EPM is widely used to evaluate anxiety-like behavior based on the natural aversion of rodents to open spaces, where increased time spent in closed arms reflects heightened anxiety (Farhaan and Haleem, 2015; Sequeira-Cordero et al., 2019) . Similarly, locomotor activity measured using an actophotometer serves as an indicator of exploratory behavior and central nervous system activity (Vijayakumar D, Suresh K, Manoharan S. 2006),

At the molecular level, chronic stress exerts profound effects on brain function, primarily through dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis. Persistent activation of the HPA axis leads to elevated glucocorticoid levels, which adversely affect the hippocampus, a key region involved in stress regulation and emotional processing. This results in neuronal

atrophy, reduced synaptic plasticity, and apoptosis (Chen et al., 2013) . Several genes are implicated in stress-induced neurobiological changes. For instance, **NR3C1**, encoding the glucocorticoid receptor, plays a pivotal role in feedback regulation of the HPA axis, while **BDNF (Brain-Derived Neurotrophic Factor)** is essential for neuronal survival and plasticity, and is often downregulated under stress conditions (Murakami et al., 2005) .

Additionally, alterations in neurotransmitter systems, particularly glutamatergic and GABAergic pathways, contribute significantly to the development of depressive disorders. The NMDA receptor, a key component of the glutamatergic system, exhibits region-specific changes under chronic stress, whereas **GABRA1**, a subunit of the GABA_A receptor, shows altered expression associated with impaired inhibitory neurotransmission (Lei and Tejani-Butt, 2010; Guofen et al., 2021). Other genes such as **FADD**, involved in apoptosis, and **CDK6**, regulating cell cycle progression, also demonstrate stress-induced dysregulation, contributing to neuronal damage and impaired neurogenesis.

L-theanine, a naturally occurring amino acid predominantly found in green tea, has gained attention for its potential neuroprotective, anxiolytic, and antidepressant properties. It is known to modulate neurotransmitter levels, enhance alpha brain wave activity, and exert a calming effect without sedation. However, its role in modulating stress-induced behavioral and molecular changes, particularly in the UCMS model, remains to be fully elucidated.

Therefore, the present study aims to evaluate the **in vivo effects of L-theanine on behavioral alterations and gene expression changes** in a UCMS-induced Wistar albino rat model. By integrating behavioral assessments with molecular analysis, this study seeks to provide a comprehensive understanding of the therapeutic potential of L-theanine in stress-related disorders.

**Materials and Methods Experimental Animals:**

Adult Wistar albino rats were used for the study. The animals were maintained under standard laboratory conditions with controlled temperature, humidity, and a 12-hour light dark cycle, with free access to food and water.

**Induction of Chronic Stress:**

Chronic stress was induced using the **Unpredictable Chronic Mild Stress (UCMS)** model, wherein animals were exposed to a series of mild and unpredictable stressors over a defined period to simulate depressive-like conditions.

**Drug Treatment:**

Animals were divided into different experimental groups, including control, stress, and treatment groups. L-theanine was administered to evaluate its therapeutic effects, while fluoxetine was used as a standard reference drug.

**Behavioral Assessment:**

Anxiety-like behavior was assessed using the **Elevated Plus Maze (EPM)**, where the time spent in open and closed arms was recorded. Locomotor activity was evaluated using an **actophotometer**, which measures movement based on interruptions of infrared light beams.

**Gene Expression Analysis:**

Total RNA was isolated from tissue samples using the TRIR method. RNA purity and concentration were assessed spectrophotometrically at 260/280 nm. Complementary DNA (cDNA) was synthesized and gene expression analysis was performed using reverse transcription–polymerase chain reaction (RT-PCR). The expression of key genes, including **BDNF, GABRA1, CRHR2, FADD, CDK6,** and **NR3C1**, was analyzed.

**Statistical Analysis:**

Data were expressed as mean ± standard error. Statistical significance between groups was determined using one-way ANOVA followed by appropriate post-hoc tests, with $p < 0.05$ considered significant.

**Ethical Approval:**

All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) of Saveetha Medical College and Hospital (Approval No: SU/CLAR/RD/035/2017; dated 23–24 August 2017, Chennai, Tamil Nadu). Animal care, handling, and disposal were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**Results:**

The results of the present study demonstrate that chronic unpredictable mild stress (UCMS) induced significant anxiety-like behavior, reduced locomotor activity, and marked alterations in gene expression, all of which were effectively ameliorated by treatment with L-theanine and Fluoxetine. In the Elevated Plus Maze test, UCMS rats exhibited a significant decrease in time spent in the open arms and a corresponding increase in time spent in the closed arms ($p < 0.05$), indicating heightened anxiety. Treatment with L-theanine and Fluoxetine reversed these effects; notably, L-theanine showed a significantly greater increase in open arm time and a reduction in closed arm time compared to Fluoxetine ($p < 0.05$), suggesting superior anxiolytic activity. Similarly, in the actophotometer test, UCMS exposure led to a decline in locomotor activity across days, while both treatment groups demonstrated gradual and statistically significant improvement ($p < 0.001$), with L-theanine showing consistent restoration comparable to or better than Fluoxetine. At the molecular level, UCMS resulted in downregulation of BDNF, CRHR2, CdK6, and Nr3c1 genes, along with upregulation of FADD and GABRA1, reflecting impaired neuroplasticity, altered stress response, increased apoptosis, and neurotransmitter imbalance. Treatment with L-theanine and Fluoxetine significantly normalized these alterations, with L-theanine showing comparatively better upregulation of BDNF and stronger regulation of apoptotic and stress-related genes. The negative

control groups did not exhibit any significant changes in gene expression. Overall, these findings indicate that L-theanine effectively reverses stress-induced behavioral and molecular changes and may serve as a promising antidepressant agent with efficacy comparable to or exceeding that of Fluoxetine.

**Table 6.1: Effect of L-theanine on Behavioural Analysis – Actophotometer Locomotor Activity**

| S.No. | Parameter | Groups | Mean | SE | Statistics |
|---|---|---|---|---|---|
| 1 | Actophotometer Locomotor Acttivity(APLA-0) | Control | 430.6 | 20 7 | Given in Figure 5.7 |
| | | FL | 402.5 | 4.1 | |
| | | LT | 406.8 | 2.0 | |
| | | Stress | 415.3 | 3.8 | |
| | | Stress+FL | 415.0 | 2.7 | |
| | | Stress+LT | 413.1 | 2.9 | |
| 2 | Actophotometer Locomotor Acttivity(APLA-1) | Control | 438.0 | 8.2 | |
| | | FL | 405.3 | 1.9 | |
| | | LT | 396.6 | 2.6 | |
| | | Stress | 391.6 | 3.3 | |
| | | Stress+FL | 396.8 | 1.4 | |
| | | Stress+LT | 402.2 | 1.4 | |
| 3 | Actophotometer Locomotor Acttivity(APLA-2) | Control | 424.6 | 3.6 | |
| | | FL | 405.5 | 3.9 | |
| | | LT | 393.6 | 3.5 | |
| | | Stress | 342.3 | 8.0 | |
| | | Stress+FL | 376.8 | 2.4 | |
| | | Stress+LT | 365.6 | 3.1 | |
| 4 | Actophotometer Locomotor Acttivity(APLA-3) | Control | 429.3 | 2.6 | |
| | | FL | 415.3 | 4.1 | |
| | | LT | 381.1 | 2.5 | |
| | | Stress | 307.5 | 5.4 | |
| | | Stress+FL | 383.8 | 1.9 | |
| | | Stress+LT | 360.3 | 4.6 | |
| 5 | Actophotometer Locomotor Acttivity(APLA-4) | Control | 433.5 | 5.5 | |
| | | FL | 418.6 | 3.4 | |
| | | LT | 408.8 | 4.3 | |
| | | Stress | 296.1 | 3.1 | |
| | | Stress+FL | 384.0 | 3.0 | |
| | | Stress+LT | 366.6 | 3.3 | |

**Table 6.2: Primer sequences for real-time polymerase chain reaction assay**

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| BDNF | CAGGGCAGTTGGACAGTCAT | TACGCAAACGCCCTCATTCT |
| CRHR2 | GGC CTC AAG GAT CAA CTA CTC | ACGTATGGGACGGGATAGTAA |
| FADD | CCAAACAAGTGCAAGAGCCC | AGGATTGCAGAGTGAGCCAC |
| CDK6 | GCT GAC CAG CAG TAC GAA TG | GCA CAC ATC AAA CAA CCT GAC |
| GABRA | GCCCTCCCAAGATGAACTTA | AGTTACACGCTCTC CCAAGC |
| NR3C1 | GGGACACGAATGAGGATTG | CACACTGCTGGGACTTGA |

BDNF – Brain Derived Neurotropic Factor gene, CRHR2 – Corticotrophin Releasing Hormone Receptor 2, FADD – FAS-Associated via Death Domain (genetics), CDK6 – Cyclin Dependent Protein Kinase 6 gene, GABRA1- gamma-aminobutyric acid type A receptor alpha1 subunit, NR3C1 - Nuclear Receptor Subfamily 3, group C, member 1.

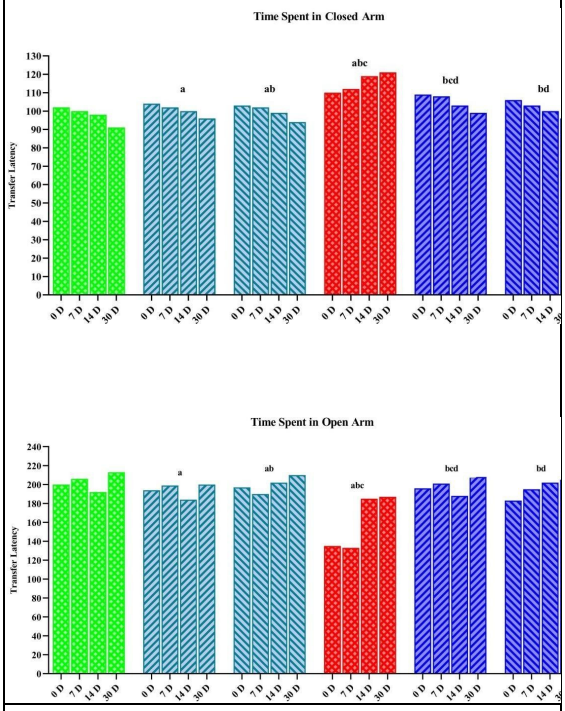



Figure 6.1: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on the Transfer Latency of Time spent in closed arm and Time spent in Open Arm by Elevated Plus Maze in rats.

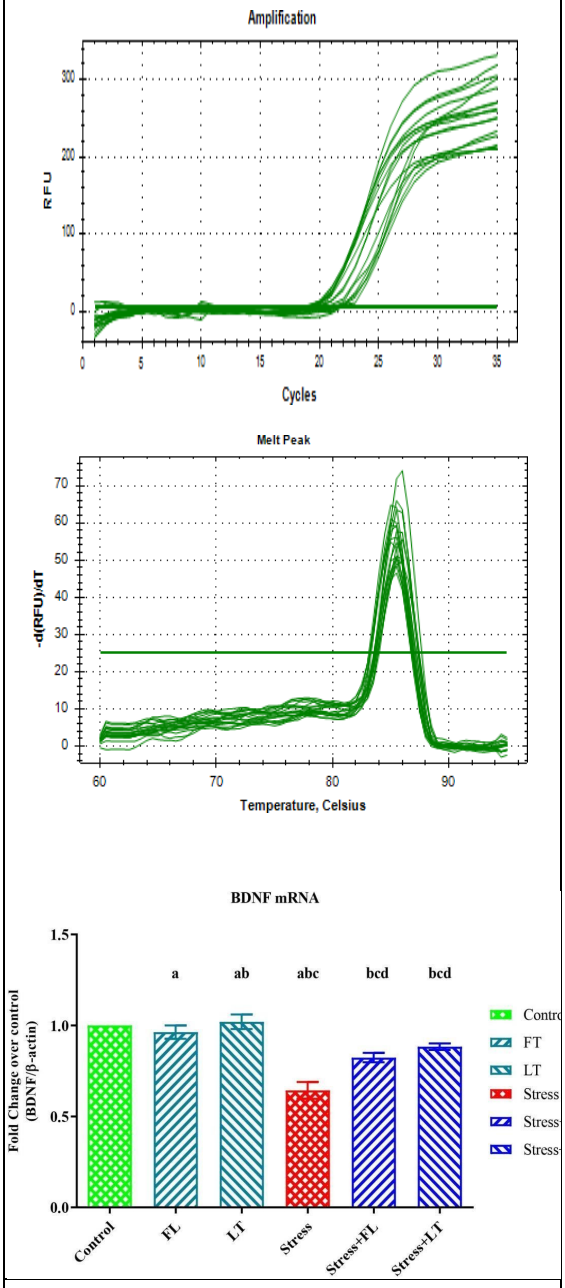
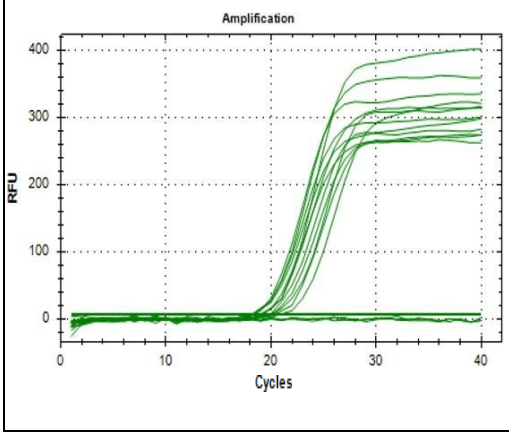



Figure 6.3: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on BDNF expression in rats.



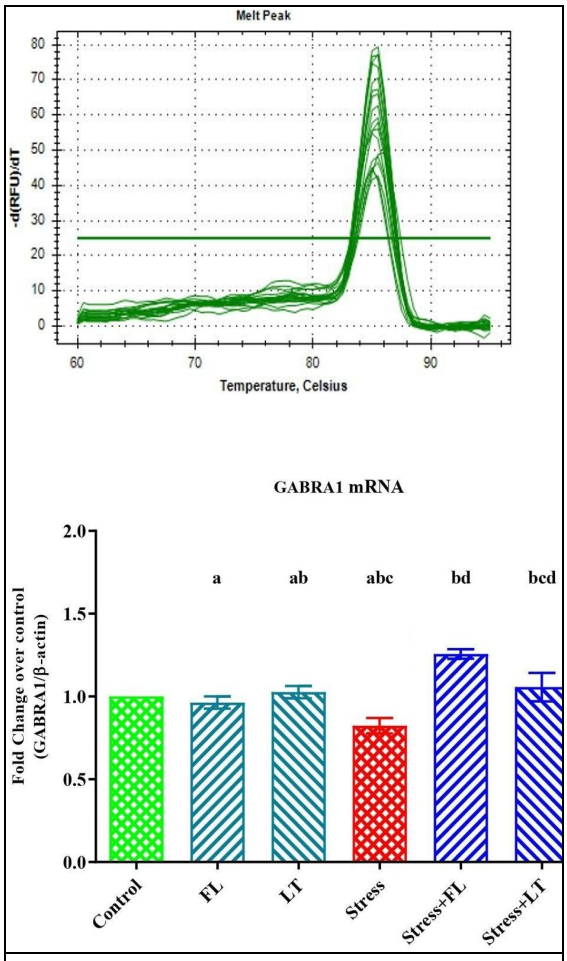



Figure 6.4: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on GABRA1 expression in rats.

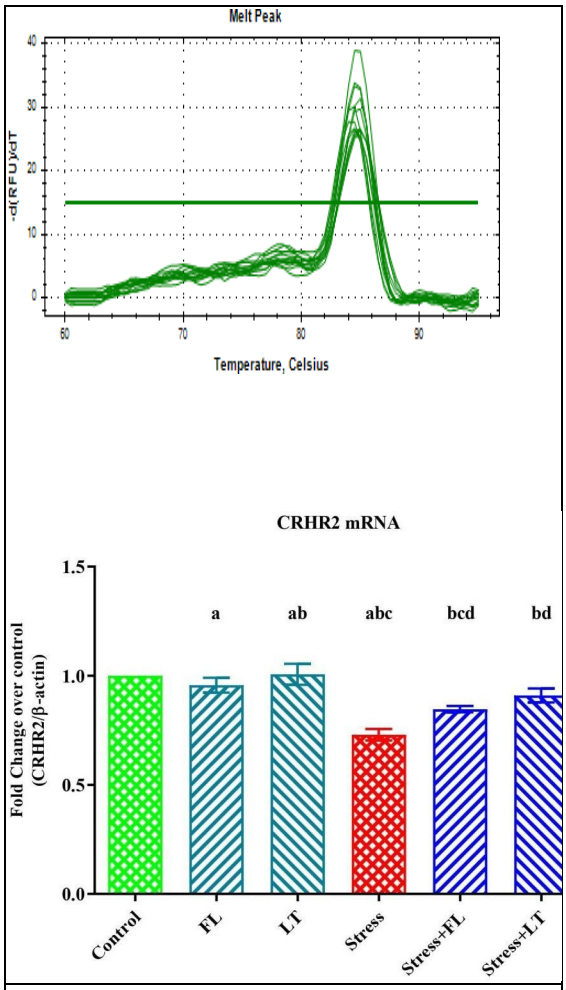
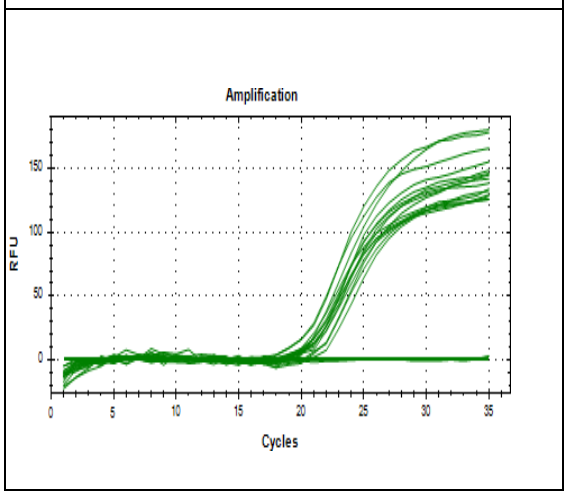
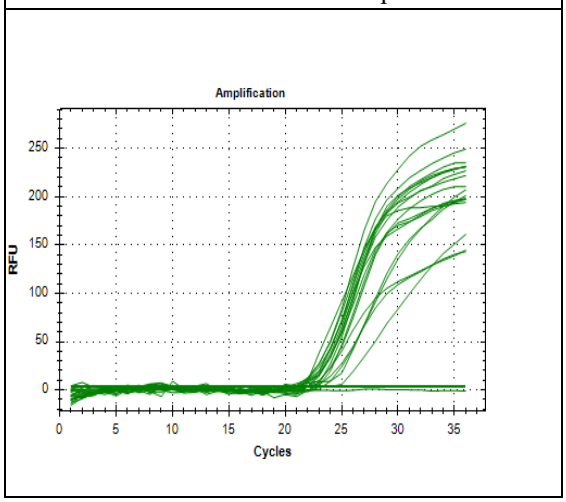



Figure 6.5: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on CRHR2 expression in rats.



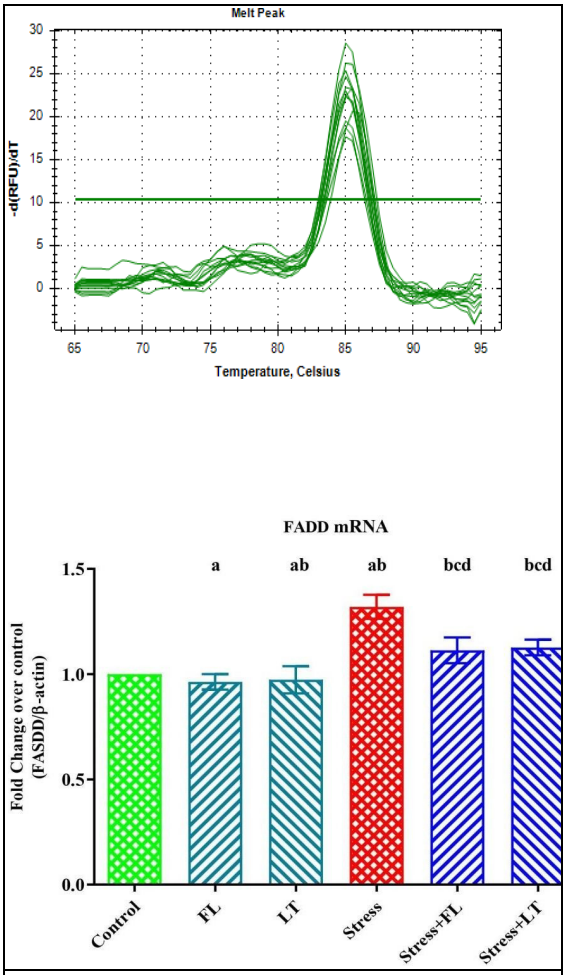



Figure 6.6: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on FADD expression in rats

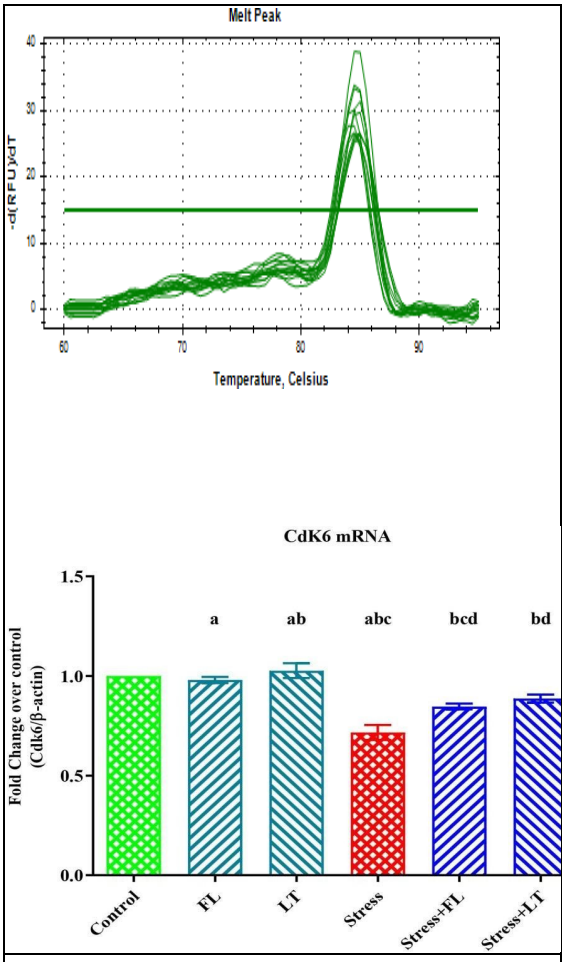
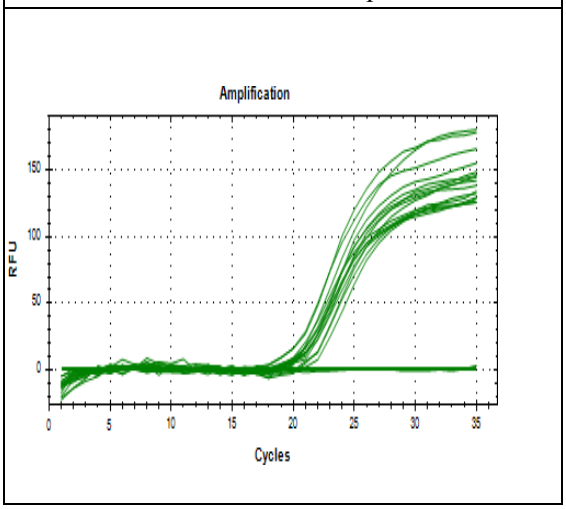
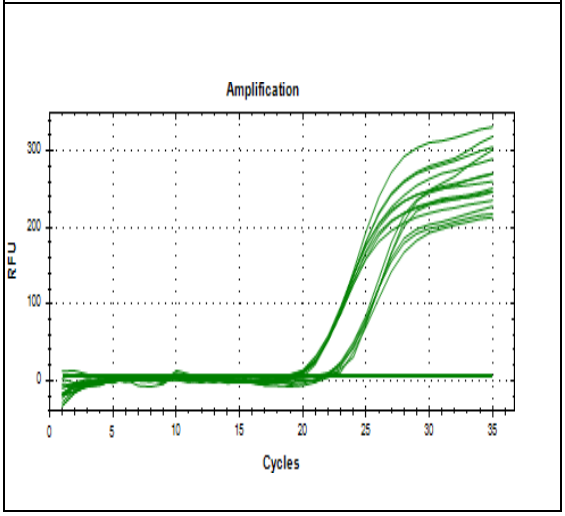



Figure 6.7: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on CdK6 expression in rats.



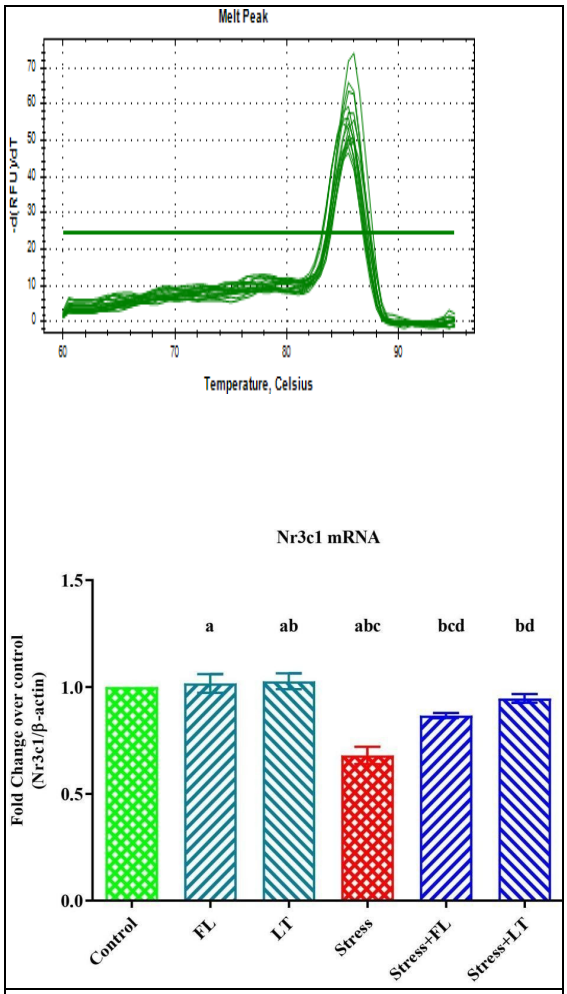



Figure 6.8: Effect of L-theanine (LT) and Fluoxetine (FL) on Unpredictable chronic mild stress induced stress on Nr3C1 expression in rats.

**Discussion:**

The Elevated Plus Maze (EPM) test, a well-established behavioral paradigm for assessing anxiety in rodents, was employed in the present study to evaluate anxiety-like behavior induced by chronic unpredictable mild stress (UCMS). The findings revealed that UCMS exposure significantly reduced the time spent in the open arms while increasing the time spent in the closed arms, indicating heightened anxiety levels. This behavioral alteration reflects the impact of chronic stress on central neural circuits involved in emotional regulation, particularly within the limbic system. Treatment with L-theanine and Fluoxetine effectively reversed these behavioral changes, demonstrating their anxiolytic potential. Notably, L-theanine exhibited a comparatively greater effect in restoring normal exploratory behavior, suggesting its superior efficacy in mitigating stress-induced anxiety. These observations may be attributed to the modulation of neurotransmitter systems and restoration of homeostatic balance disrupted during chronic stress exposure (Pellow et al., 1985; Walf and Frye, 2007), (Tilbrook, A.J., Turner, A.I. and Clarke, I.J., 2000). At the molecular level, chronic stress resulted in significant dysregulation of genes associated with neuroplasticity, stress response, apoptosis, and neuronal survival. Specifically, the genes **Nr3c1**, **CRHR2**, **CdK6**, and **BDNF** were markedly downregulated in the UCMS group. The downregulation of **BDNF**, a key neurotrophic factor, suggests impaired synaptic plasticity and neuronal survival, which are critical in the pathophysiology of depression (Duman and Monteggia, 2006). Similarly, reduced expression of **Nr3c1**, which encodes the glucocorticoid receptor, indicates disruption of hypothalamic–pituitary–adrenal (HPA) axis regulation, leading to impaired stress adaptability (Pariante and Lightman, 2008). The decreased expression of **CRHR2** further reflects altered stress response signaling, while suppression of **CdK6** suggests impaired cell cycle progression and reduced neuronal proliferation (Ueki S, Watanabe S, Yamamoto T Et al. 1987).

Conversely, chronic stress induced significant upregulation of **FADD** and **GABRA1** genes. The increased expression of **FADD** (Fas-Associated protein with Death Domain) is associated with enhanced apoptotic signaling, leading to neuronal cell death, particularly in the hippocampus. This finding is consistent with previous studies by Chen et al. (2013) and Chen et al. (2019), which demonstrated that chronic stress elevates FADD expression, contributing to hippocampal apoptosis and impaired neuronal function. Similarly, upregulation of **GABRA1**, a subunit of the GABA_A receptor, indicates alterations in inhibitory neurotransmission, which may contribute to anxiety and depressive-like behaviors (Ulrich-Lai, Y.M. and Herman, J.P., 2009). The consistency of these findings with earlier reports strengthens the validity of the present study.

Importantly, treatment with L-theanine and Fluoxetine significantly normalized these stress-induced molecular alterations. The restoration of **BDNF**, **Nr3c1**, **CRHR2**, and **CdK6** expression suggests improved neuroplasticity, enhanced stress response regulation, and recovery of neuronal growth mechanisms. Additionally, the downregulation of **FADD** following treatment indicates reduced apoptotic activity, while normalization of **GABRA1** expression reflects stabilization of inhibitory neurotransmission. Among the two treatments, L-theanine demonstrated a more pronounced effect in modulating these molecular pathways, suggesting its potential role as a neuroprotective and anti-apoptotic agent

The behavioral improvements observed in the EPM test can thus be correlated with the underlying molecular changes, indicating that L-theanine exerts its anxiolytic and antidepressant effects through a multifaceted mechanism involving modulation of the HPA axis, enhancement of neurotrophic support, regulation of neurotransmitter systems, and inhibition of stress-induced apoptosis. These findings support the potential of L-theanine as an effective therapeutic

agent for stress-related neuropsychiatric disorders, with efficacy comparable to conventional antidepressants such as Fluoxetine.

**Conclusion:**

The present study demonstrates that chronic unpredictable mild stress (UCMS) induces significant anxiety-like behavior, reduced locomotor activity, and marked dysregulation of genes associated with neuroplasticity, stress response, apoptosis, and neurotransmission. Behavioral assessments using the Elevated Plus Maze and actophotometer confirmed that stress exposure leads to increased anxiety and decreased exploratory and locomotor activity. At the molecular level, UCMS resulted in downregulation of **BDNF, Nr3c1, CRHR2, and CdK6**, along with upregulation of **FADD and GABRA1**, indicating impaired neuroplasticity, altered hypothalamic–pituitary–adrenal (HPA) axis function, increased apoptotic signaling, and neurotransmitter imbalance.

Treatment with L-theanine and Fluoxetine effectively reversed these behavioral and molecular alterations. Notably, L-theanine exhibited a comparatively superior effect in reducing anxiety-like behavior, improving locomotor activity, and normalizing gene expression, particularly by enhancing BDNF levels and reducing FADD-mediated apoptotic pathways. These findings suggest that L-theanine exerts its therapeutic effects through modulation of neurotrophic signaling, stress response mechanisms, and neuronal survival pathways.

In conclusion, L-theanine demonstrates significant anxiolytic and antidepressant-like properties, with efficacy comparable to or exceeding that of Fluoxetine in certain parameters. Therefore, L-theanine holds promise as a potential therapeutic agent for the management of stress-related neuropsychiatric disorders.

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