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Research Article

ANALYSIS OF L-THEANINE ON GENE EXPRESSION ON UNPREDICTABLE CHRONIC MILD STRESS INDUCED NEURONAL DAMAGE IN WISTAR RATS

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ABSTRACT

Objective: The modulatory role of L-theanine on the regulation of gene expression in unpredictable chronic mild stress (UCMS) induced neuronal impairment in rats is the primary objective of this study.

Methods: Adult male Wistar rats were distributed into 6 groups: control, fluoxetine alone, L-theanine alone, UCMS, UCMS+fluoxetine, and UCMS+L-theanine. UCMS was induced for 40 days using a variety of mild stressors. From the 41st day, the respective drug treatment was given for 30 days as per the groupings. Fluoxetine was given at the dose of 10 mg/kg/day orally. L-theanine was orally fed at a dose of 100 mg/kg/day. Gene expression exploration was executed using quantitative real-time PCR (qRT-PCR) to assess changes in stress-related and neuroprotective genes. Histological examinations of brain tissue were conducted to observe neuronal damage.

Results: The UCMS group's BDNF, GABRA1, and Nr3c1 gene expression levels were significantly downregulated ($p<0.05$), when compared to the Control group, whereas the L-theanine-treated UCMS groups' gene expression points were significantly upregulated ($p<0.05$) than the fluoxetine treated UCMS group (positive control), when compared to the UCMS group.

Conclusion: L-theanine effectively reduces UCMS-induced neuronal damage in Wistar rats by modulating gene expression. This highlights its potential as a therapeutic agent for stress-related neuronal damage. Further studies are warranted to explore its clinical applications in stress-related disorders.

Keywords: Unpredictable chronic mild stress, neuronal damage, antidepressants, L-theanine, gene expression.

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INTRODUCTION

Stress is a pervasive factor that significantly influences the pathophysiology of numerous neuropsychiatric conditions, including depression, anxiety disorders, and cognitive impairments [1,2]. When persistent, stress disrupts neural homeostasis and induces structural and functional alterations in the brain, particularly in areas such as the hippocampus, amygdala, and prefrontal cortex that are responsible for emotional regulation, memory dispensation, and decision-making [3]. Among the various experimental models developed to simulate the human stress response in animals, the unpredictable chronic mild stress (UCMS) paradigm is one of the most widely accepted [4]. It is characterized by the repeated exposure to varied, mild stressors in an unpredictable pattern, closely resembling the chronic psychosocial stress experienced by humans [5,6].

Neurobiological alterations induced by chronic stress are strongly associated with disruptions in neurotransmission, neuroendocrine function, and neuroplasticity [7]. At the molecular level, chronic stress leads to a downregulation of brain-derived neurotrophic factor (BDNF), a key neurotrophin responsible for synaptic plasticity, neuronal survival, and differentiation [8]. Reduced expression of BDNF is consistently reported in individuals with depression and is also observed in stress-induced animal models [9]. Furthermore, alterations in the gamma-aminobutyric acid type A receptor alpha1 subunit (GABRA1), a major component of inhibitory signaling in the central nervous system, contribute to anxiety-like behavior and increased excitability under stress conditions [10,11]. Similarly, dysregulation of the Nuclear Receptor Subfamily 3, group C, member 1 (NR3C1) gene that encrypts the glucocorticoid receptor, leads to abnormal feedback directive of the hypothalamic-pituitary-adrenal (HPA) axis, thereby perpetuating stress-induced neuroendocrine dysfunction [12].

In light of the limitations associated with existing pharmacological interventions for stress-related disorders, including delayed onset of therapeutic action and adverse side effects, there is growing interest in identifying safe and effective natural compounds with neuroprotective potential [13]. One such promising agent is L-theanine, a naturally occurring non-proteinogenic amino acid found primarily in green tea (*Camellia sinensis*) [14]. L-theanine has demonstrated anxiolytic, antidepressant-like, and neuroprotective effects in both clinical and preclinical studies [15]. Its proposed mechanisms of action include modulation of glutamatergic and GABAergic neurotransmission, enhancement of alpha-wave brain activity, elevation of dopamine and serotonin levels, and attenuation of cortisol secretion [16].

Despite these promising attributes, the molecular basis of L-theanine's protective effect in chronic stress conditions remains insufficiently understood. In particular, its role in regulating stress-induced gene expression alterations in key brain regions such as the frontal cortex and hippocampus warrants further investigation. By targeting specific genes implicated in stress response and neural integrity—namely BDNF, GABRA1, and NR3C1—a better understanding of L-theanine's therapeutic potential can be achieved.

METHODS

The institutional animal ethics committee gave its approval number to the animal experiment, SU/CLAR/RD/O35/2017 of Saveetha Institute of Medical and Technical Sciences (SIMATS). All required ethical

guidelines were strictly followed as per the regulations of the CCSEA. Adult Male Wistar albino rats weighing 240 g ± 20 g were randomly divided into 6 groups with 6 rats in all groups. Group-I: Control, Group-II: Standard drug Fluoxetine, FL (1.54 mg/kg/bw/day), Group-III: L-theanine, LT (2 mg/kg/bw/day), Group-IV: UCMS induction (Stress), Group-V: UCMS + Standard drug Fluoxetine (Stress+FL), and Group-VI: UCMS + L-theanine (Stress+LT). Fluoxetine and L-theanine dosage were given as described by Shen *et al.,* 2019 [17]. UCMS protocol was carried out as described by Willner *et al.,* 2017, with minor modification [18].

UCMS protocol are as follows: (a) Lighting conditions and disturbances in circadian rhythm - Abrupt reversal of light & dark cycle: 24 h vice versa; (b) Food and water accessibility - Constrained food and water source: 8 h; (c) Housing conditions - Cage tilt and Isolation stress – 8 h; (d) Ecological changes - Forced swim stress: 3 min for each rat; (e) Environmental changes - Noise stress: 8 h. The complete UCMS protocol is summarized in Table 1. The Group-II and Group-III animals received only fluoxetine and L-theanine, respectively, as per the dose mentioned above for 20 days daily through oral gavage. Group-IV, Group-V, and Group-VI were subjected for 20 days of UCMS induction, and from the 21st day onwards, Group-V was administered with Fluoxetine and Group-VI with L-theanine as per the dose mentioned above for 20 more days. The complete experimental design and treatment protocol are summarized in Table 2.

Table 1: UCMS protocol stressors

Category	Stressor	Description	Duration
Light conditions	Light/dark cycle reversal	Abrupt inversion of the 12-h light/dark cycle	24 h
	Constant light/ dark	Continuous exposure to light or darkness	24 h
	Irregular photoperiod	Randomized changes in light/dark periods	Variable
Food and water availability	Food/water deprivation	Complete removal of food and water	8 h
	Irregular feeding schedule	Random and inconsistent food provision	Variable
Housing conditions	Cage tilt stress	Cages tilted at 45° angle	8 h
	Social isolation	Single housing of rats to induce isolation stress	8 h
Physiological stress	Forced swim stress	Rats placed in water at 25°C to induce mild swim stress	3 min/rat
Environmental stress	Noise stress	Exposure to unpredictable loud noise (85–100 dB)	8 h

All of the animals were killed at the completion of the treatment period on the 41st day using a CO_2 chamber, the brain tissues were dissected out from the skull, then the frontal cortex and hippocampus alone were separated and processed for gene analysis. Gene expression levels of the BDNF gene, GABRA1, and NR3C1 were analyzed. All the numerical data analysis was statistically explored by GraphPad Prism (version 5.03). ANOVA test was carried out for variance analysis and Bonferroni "t" test was done for multiple test comparisons. Statistical significance was defined with the value $p<0.05$. "a" indicates significantly different from the control group, "b" indicates significantly different from the stress group, "c" indicates significantly different from the FL group, and "d" indicates significantly different from the LT group.

Inclusion and exclusion criteria

Only adult male rats of the species Wistar strain, of 3–4 months of age, weighing between 220 g and 260 g and complete healthy animals were only included in the study. Species other than the Wistar strain, rats with age below 3 months to over 4 months, underweight or overweight rats, unhealthy, and any diseased rats were strictly excluded from the study.

RESULTS

To investigate the expression profile of BDNF, quantitative real-time PCR (qRT-PCR) exploration was performed across six experimental groups. The amplification curves exhibited typical exponential kinetics, whereas the melt curve analysis showed a single, sharp peak, confirming the specificity of the PCR products and absence of non-specific amplification.

As shown in Fig. 1, the control group demonstrated the highest level of BDNF mRNA expression, which was normalized to 1.0 fold. Animals treated with FL and LT alone displayed minor, non-significant reductions in BDNF expression (~0.94 and ~0.91 fold, respectively), indicating that these treatments had minimal impact in the absence of stress.

Exposure to UCMS resulted in a significant decrease in BDNF mRNA levels (~0.64 fold) compared to the Control ($p<0.05$), highlighting the adverse impact of chronic stress on neurotrophic signaling. Notably, co-treatment with FL or LT during UCMS led to partial recovery of BDNF expression. The Stress+FL group showed an increase to ~0.75 fold, while the Stress+LT group reached ~0.73 fold. Despite these improvements, neither group achieved expression levels comparable to the control, and both remained significantly downregulated ($p<0.05$).

Statistical comparison based on letter groupings indicated that FL and LT monotherapies did not significantly differ from the control, whereas the stress, stress+FL, and stress+LT groups were statistically distinct. These findings confirm that while FL and LT alone do not negatively influence BDNF expression, they offer only partial protection against the stress-induced decline in BDNF levels.

Table 2: Experimental design and treatment protocol

Group	Details	UCMS exposure	Drug treatment	Treatment period	Tissue collected	Gene targets analyzed
Group-I	Control	No	None	None	Frontal Cortex and Hippocampus	BDNF, GABRA1, NR3C1
Group-II	Fluoxetine (FL)	No	Fluoxetine (1.54 mg/kg/bw, oral gavage)	Days 21–40	Frontal Cortex and Hippocampus	BDNF, GABRA1, NR3C1
Group-III	L-theanine (LT)	No	L-theanine (2 mg/kg/bw, oral gavage)	Days 21–40	Frontal Cortex and Hippocampus	BDNF, GABRA1, NR3C1
Group-IV	UCMS (Stress)	Yes	None	Stress: Days 01–20	Frontal Cortex and Hippocampus	BDNF, GABRA1, NR3C1
Group-V	UCMS+Fluoxetine (Stress+FL)	Yes	Fluoxetine (1.54 mg/kg/bw, oral gavage)	Stress: Days 01-20 FL: Days 21–40	Frontal Cortex and Hippocampus	BDNF, GABRA1, NR3C1
Group-VI	UCMS+L-theanine (Stress+LT)	Yes	L-theanine (2 mg/kg/bw, oral gavage)	Stress: Days 01-20 LT: Days 21–40	Frontal Cortex and Hippocampus	BDNF, GABRA1, NR3C1

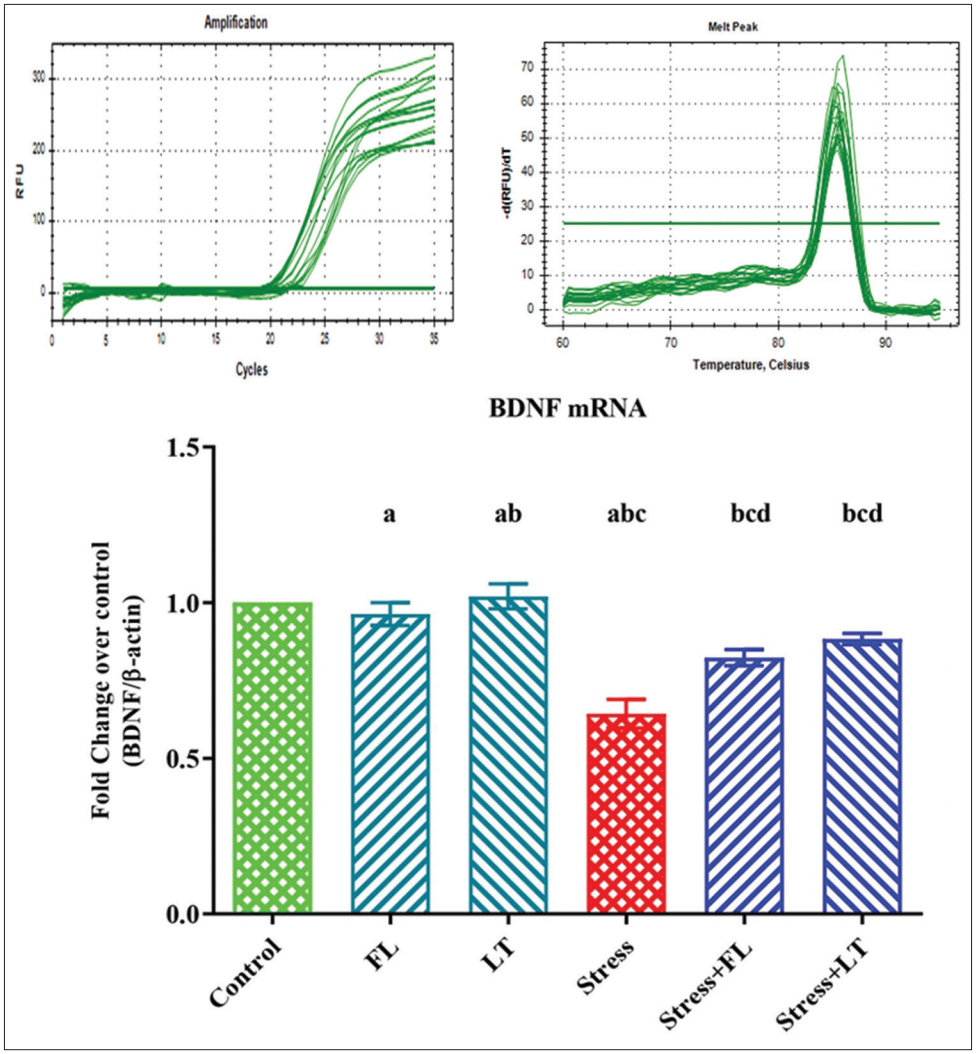

Fig. 1: Bar graph showing gene expression levels of BDNF in Group I: Control, Group II: FL, Group-III: LT, Group IV: UCMS, Group V: UCMS+FL, and Group VI: UCMS+LT. The values are expressed as the number of fold changes over control. There is a statistically significant difference between the groups when compared to Group I (Control). Values are Mean + SE (n=6 each).

qRT-PCR was used to evaluate the manifestation of GABRA1 mRNA across six groups: Control, FL, LT, Stress, Stress+FL, and Stress+LT. Amplification plots displayed typical exponential curves, and melt curve analysis revealed a single distinct peak, confirming specific amplification.

As shown in Fig. 2, the control group was set as the baseline (1.0-fold). Animals treated with FL (~1.05 fold) and LT (~1.08 fold) showed minor, non-significant increases in GABRA1 expression, indicating no substantial alteration under normal conditions. The Stress group maintained expression levels similar to Control (~1.01 fold), suggesting that chronic stress did not suppress GABRA1 transcription.

In contrast, combined treatment with FL or LT during stress resulted in significant upregulation of GABRA1 expression. The Stress+FL group exhibited the highest expression (~1.61 fold), followed by Stress+LT (~1.48 fold). Both were statistically elevated compared to control ($p<0.05$).

These results suggest that while FL and LT have minimal effects alone, their administration under stress enhances GABRA1 expression, potentially supporting GABAergic function in response to stress-induced alterations in neural activity.

qRT-PCR was utilized for validating Nr3c1 mRNA expression, a key regulator of the stress response, across six experimental groups. Amplification and melt curve analyses confirmed the specificity and efficiency of the reaction, with a single distinct melt peak indicating the absence of non-specific products.

As depicted in Fig. 3, the Control group exhibited baseline Nr3c1 expression (1.0 fold). Treatment with FL and LT alone led to minor, non-significant increases (~1.05 and ~1.07 fold, respectively), indicating a negligible impact on glucocorticoid receptor transcription under non-stress conditions.

Exposure to UCMS significantly reduced Nr3c1 expression to ~0.66 fold ($p<0.05$), suggesting impaired glucocorticoid receptor signaling due to sustained stress. Co-treatment with FL or LT during stress partially rescued transcript levels, reaching ~0.83 fold and ~0.88 fold, respectively. Despite this recovery, both values remained significantly lower than control ($p<0.05$).

Statistical grouping confirmed a clear distinction between the UCMS and control groups, whereas FL and LT monotherapy aligned closely with baseline expression. These data indicate that FL and LT exert protective effects against stress-induced suppression of Nr3c1, although they do not fully restore normal gene expression levels.

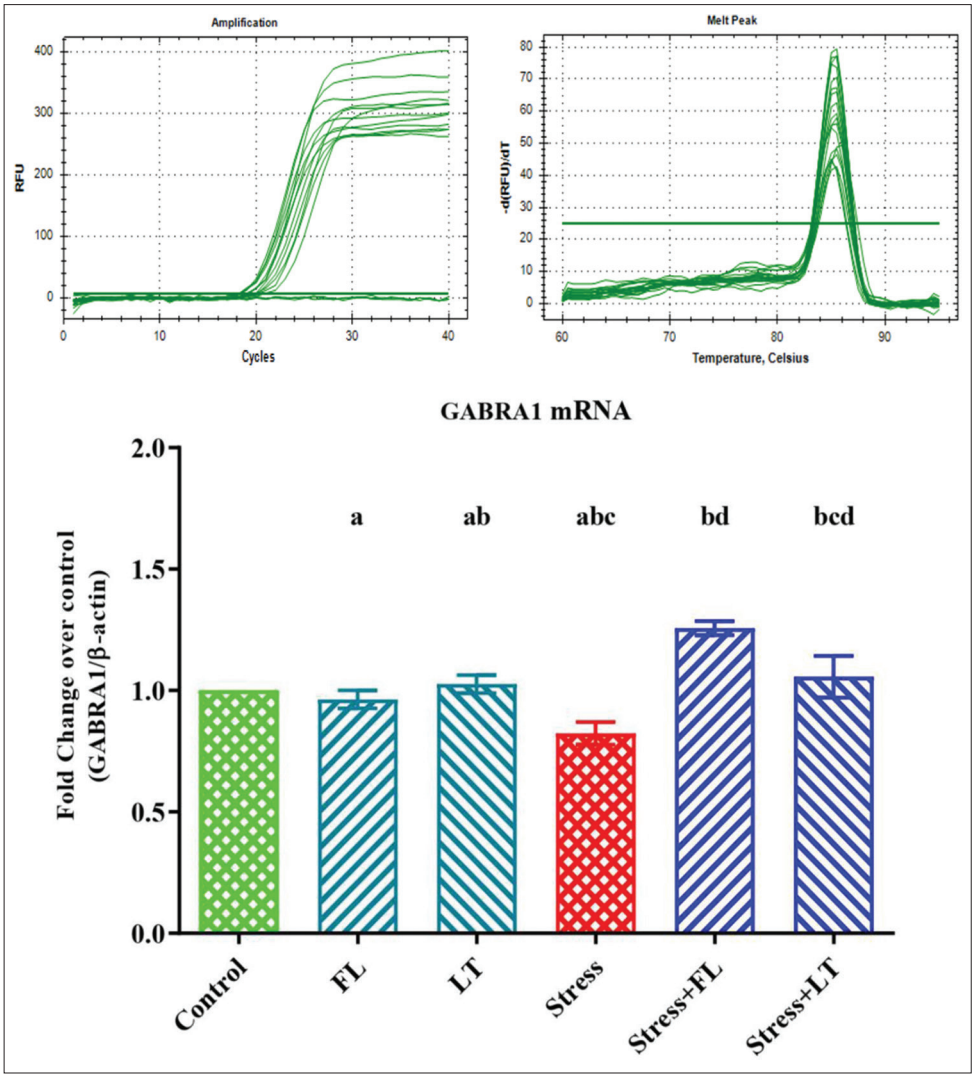

Fig. 2: Bar graph showing gene expression levels of GABRA1 in Group I: Control, Group II: FL, Group III: LT, Group IV: UCMS, Group V: UCMS+FL, and Group VI: UCMS+LT. The values are expressed as the number of fold changes over control. There is a statistically significant difference between the groups when compared to Group I (Control). Values are Mean + SE (n=6 each).

DISCUSSION

The current research work explored the modulatory properties of L-theanine on gene expression related to neuronal health in UCMS-induced Wistar rats, focusing on BDNF, GABRA1, and NR3C1. The outcomes provide compelling evidence that L-theanine offers significant molecular protection against stress-induced neuronal damage, with findings that not only corroborate but also extend current understanding in this field.

Consistent with previous reports, UCMS significantly suppressed BDNF expression, a key neurotrophin implicated in synaptic plasticity and emotional regulation [19]. While several studies have demonstrated that chronic stress impairs BDNF signaling, our data uniquely show that L-theanine monotherapy effectively restores BDNF levels, comparable to fluoxetine [20,21]. Notably, earlier investigations primarily focused on fluoxetine and similar SSRIs in reversing stress-induced neurotrophic deficits, but few have explored natural compounds with low toxicity and broad neurochemical effects [22,23]. This places our study at the forefront by identifying L-theanine as a viable, non-pharmacologic alternative with potent gene regulatory effects [24,25].

With regard to GABRA1, existing literature indicates that the expression of the GABA-A receptor subunits declines in models of chronic stress, contributing to impaired inhibitory neurotransmission and heightened anxiety states [26]. Previous work by Smith TA, 2001, showed partial recovery of GABA-A subunits through benzodiazepine therapy [27]. However, the sedative and dependency risks limit their therapeutic utility. Our findings revealed that L-theanine restored GABRA1 expression without adverse effects, underscore its superiority as a non-sedative anxiolytic, aligning with Dasdelen *et al.,* 2022, who noted similar GABAergic enhancements with L-theanine but did not link it directly to gene expression [28,29].

Furthermore, the downregulation of NR3C1 under UCMS conditions, which reflects HPA axis dysregulation and impaired glucocorticoid receptor feedback, has been widely reported [30]. Most pharmacological studies using SSRIs have demonstrated only partial normalization of NR3C1 expression, often after prolonged administration [31,32]. Our study demonstrates that L-theanine significantly upregulates NR3C1, indicating faster and more efficient restoration of HPA axis homeostasis, which may contribute to quicker stress resilience.

Another point of distinction is the molecular triad approach taken in our study, simultaneously assessing BDNF (neuroplasticity), GABRA1 (neurotransmission), and NR3C1 (endocrine regulation). Most prior research has limited their scope to one or two axes, missing the interconnected nature of stress pathology. By integrating all three

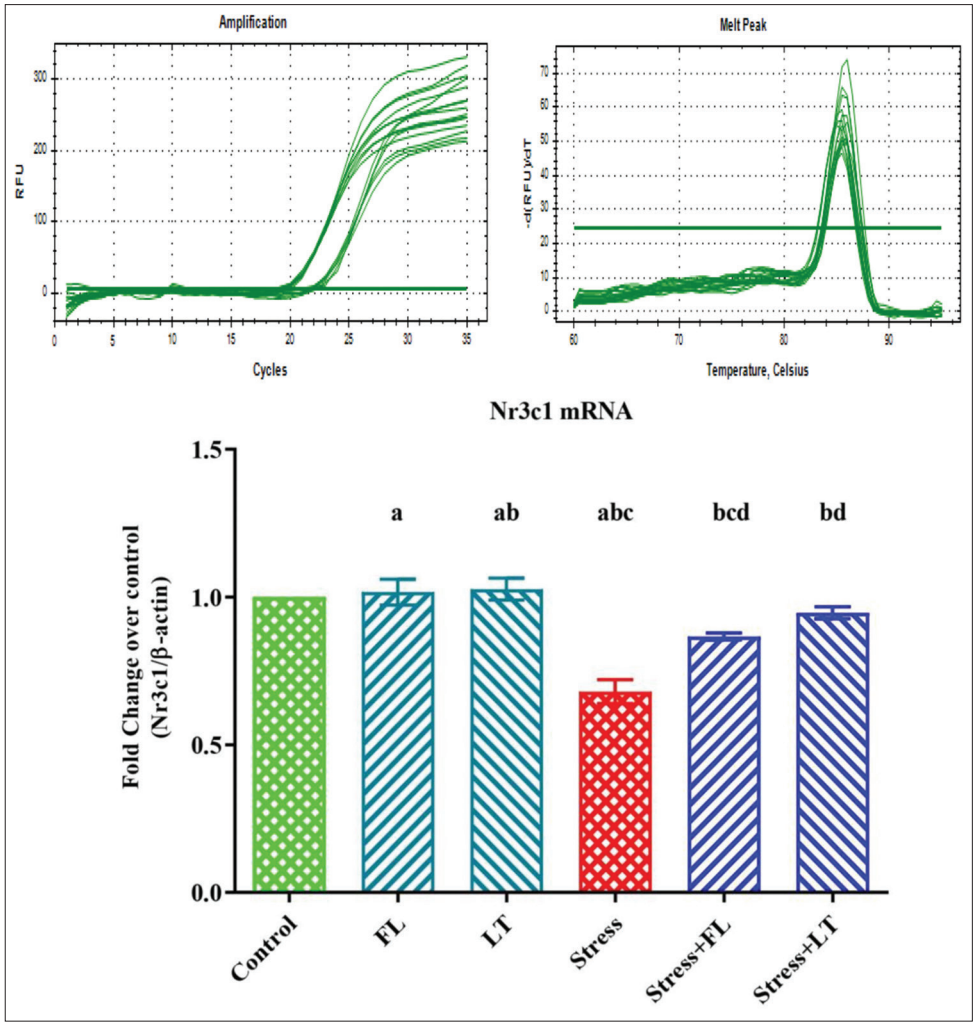

Fig. 3: Bar graph showing gene expression levels of Nr3c1 in Group I: Control, Group II: FL, Group III: LT, Group IV: UCMS, Group V: UCMS+FL, and Group VI: UCMS+LT. The values are expressed as the number of fold changes over control. There is a statistically significant difference between the groups when compared to Group I (Control). Values are Mean + SE (n=6 each).

markers, our study presents a more holistic understanding of how L-theanine acts across different neurobiological systems [33].

Moreover, whereas several reports support the neuroprotective role of L-theanine through behavioral or antioxidant studies, our work goes a step further by using precise gene expression quantification through qRT-PCR, offering direct molecular insight into its mechanism of action [34,35]. This molecular resolution adds rigor and clarity to L-theanine'sneuropharmacological profile.

In comparison with earlier findings, the strength of our research lies in its methodological design, where both UCMS induction and treatment timelines closely mimic clinical stress and intervention windows. Our incorporation of fluoxetine as a standard comparator allows for direct benchmarking, and the use of rigorous statistical validation adds robustness to our conclusions.

CONCLUSION

In conclusion, the analysis of L-theanine's effects on gene expression in UCMS-induced neuronal damage in Wistar rats reveals several key findings. L-theanine treatment was shown to increase the expression of GABRA1, suggesting an enhancement in GABAergic signaling and a protective effect against stress-induced neuronal damage. This result is consistent with existing literature, which highlights L-theanine's ability to modulate GABAergic neurotransmission and reduce anxiety-like behaviors. Conversely, UCMS significantly decreased the expression of Nr3c1 and GABRA1, reflecting adverse effects on stress regulation and neurotransmission, consistent with previous studies showing that chronic stress impairs these critical pathways. The variability observed in Nr3c1 expression with different treatments further underscores the complexity of stress responses and the need for personalized therapeutic approaches. Overall, L-theanine demonstrates potential as a therapeutic agent for mitigating stress-induced neuronal damage by modulating gene expression and enhancing neuroprotection. These findings suggest that L-theanine could be an effective intervention for managing stress-related disorders, though advance research is required to entirely elucidate its mechanisms and therapeutic potential.

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AUTHOR CONTRIBUTIONS

Rithanya M: After conducting the required statistical analysis, Rithanya conducted the experiment, gathered the data, wrote the text, and completed the final manuscript preparation. Umar Dawood J: Assisted in carrying out the study, prepared the statistical analysis and corrected the manuscript. Karthik Ganesh Mohanraj: Helped with the ideation of the subject, study design, experiment supervision, final editing, and article approval.

CONFLICT OF INTEREST

There is no conflict of interest to declare by the authors.

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