L-theanine, a Component of Green Tea Prevents 3-Nitropropionic Acid (3-NP)-Induced Striatal Toxicity by Modulating Nitric Oxide Pathway

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Abstract L-theanine is unique amino acid which readily crosses blood brain barrier and possesses neuroprotective potential against neurodegenerative disorders including Huntington disease (HD). HD is characterized by selective loss of GABAergic medium spiny neurons. 3-nitropropionic acid (3-NP) induces a spectrum of HD-like neuropathology in rat striatum and widely used as experimental tool to study HD. Therefore, the present study was intended to investigate the effect of L-theanine against 3-NP-induced striatal toxicity and to explore its possible mechanism. Rats were administered with 3-NP for 21 days. L-theanine was given once a day, 1 h prior to 3-NP treatment for 21 days and L-NAME (10 mg/kg, i.p.), NO inhibitor and L-arginine (50 mg/kg; i.p.), NO precursor were administered 1 h prior to L-theanine treatment. Body weight and behavioral observation were made on weekly basis. On the 22nd day, animals were sacrificed, and the striatum was isolated for biochemical (LPO, GSH, and nitrite), pro-inflammatory cytokines and neurochemical analysis. 3-NP treatment significantly altered body weight, locomotor activity, motor coordination, mitochondrial complex-II activity, oxidative defense, pro-inflammatory mediators, and striatal neurotransmitters level. L-theanine pre-treatment (25 and 50 mg/kg/day, p.o.) significantly prevented these alterations. In addition, concurrent treatment of L-NAME with L-theanine (25 mg/kg/day, p.o.) significantly enhanced protective effect of L-theanine (25 mg/kg/day, p.o.) whereas concurrent treatment of L-arginine with L-theanine (50 mg/kg/day, p.o.) significantly ameliorated the protective effect of L-theanine (50 mg/kg/day, p.o.). The neuroprotective potential of L-theanine involves inhibition of detrimental nitric oxide production and prevention of neurotransmitters alteration in the striatum.

Keywords 3-Nitropropionic acid · Catecholamines · Huntington's disease · GABA · Glutamate · Oxidative stress · L-theanine

Abbreviations

3-NP	3-Nitropropionic acid
5-HIAA	5-Hydroxy 3-Indole acetic acid
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	Homovanillic Acid
HD	Huntington's disease
IL	Inter leukin
MSNs	Medium spiny neurons
NMDA	N-methyl d-aspartate
NMDAR	N-methyl D-aspartate receptor
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase

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Introduction

Gamma-ethylamino-L-glutamic acid (L-theanine) is a biologically active amino acid obtained from *Camellia sinensis*. It readily crosses the blood-brain barrier, which enables its various neuroprotective effects on the brain [1]. L-theanine has been reported to increase brain dopamine, serotonin, GABA levels and exhibit micromolar affinities for

AMPA, kainate, and NMDA receptors, which enables its mood enhancing and relaxation property. Thus, L-theanine displays a neuropharmacology, suggesting it to be a possible neuroprotective, stress reducing, and antioxidant agent. Hence, it warrants further investigation in animals and humans.

Huntington's disease (HD) is a hyperkinetic neurodegenerative movement disorder that has no cure. Pathologically, HD is characterized by preferential loss of GABAergic medium spiny neurons (MSNs) in the striatum and deep layers of the cortex. The clinical profile of HD includes motor, cognitive, and psychiatric dysfunction [2–4]. Molecular assessments during disease progression show neurotransmitters alteration, excitotoxicity, oxidative stress, mitochondrial dysfunction, and neuroinflammation [5–7]. Altered level of neurotransmitters specifically dopamine (DA) in the striatum contributes to the behavioral alteration, impaired motor coordination, and striatal dysfunctioning [8]. Recent neurochemical studies have revealed increase in dopamine in the early stages of the disease while postmortem studies of late-stage HD patients showed reduced levels of caudate dopamine and its metabolites (HVA and DOPAC) [8]. Moreover, this alteration in DA level in HD is responsible for glutamate mediated excitotoxic cell neuronal death [8]. Overactivation of N-methyl-D-aspartate receptors (NMDARs) is responsible for striatal excitotoxicity, specifically through NR2B subunit [9]. Serotonin has been reported to affect striatal DA release in a state-dependent manner associated with the conditional involvement of various 5-HT receptors but the physiological relevance of this mechanism is far from clear [10]. Many lines of evidence suggest that oxidative stress resulting in increased reactive oxygen species (ROS) generation and neuro-inflammation play a pivotal role in the age-associated cognitive decline and neuronal loss in neurodegenerative diseases including HD. Thus, promising future treatment of fatal neurodegenerative diseases like HD depends on availability of effective brain permeable, free radical scavenger neuroprotective drugs that would prevent the progression of neurodegeneration.

Nitric oxide (NO) is an unconventional transmitter molecule in the nervous system and is well implicated in pathophysiology of several neurological disorders including HD [11]. NO is synthesized from L arginine by nitric oxide synthase (NOS). There are three types of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). 3-NP intoxication significantly increased the activity of NOS in the striatum, and this activation is closely linked to NMDA receptor stimulation. The presence of high NO concentrations following NMDA receptor activation results in the formation of detrimental peroxynitrite [12].

3-Nitropropionic acid (3-NP) is a well known experimental model to study HD and associated neuropsychiatric problems. 3-NP causes selective degeneration of GABAergic MSNs in the striatum through inhibition of mitochondrial complex II and produces neuronal death or brain lesions in rodents as observed in HD patients [3, 4, 7]. Given the neuroprotective and neuromodulatory potential of L-theanine, we hypothesize that L-theanine would prevent against 3-NP-induced behavioral, biochemical, and neurochemical alteration in rats.

Material and Methods

Experimental Animals

Male adult Wistar rats at age of 4–5 months (200–250 g) were obtained from central animal house of I.S.F. College of Pharmacy, Moga, Punjab (India). Animals were housed on 12-h light/12-h dark cycle for at least 1 week prior to the start of experiment in polyacrylic cages (3 rats per cage) with free access to food and water. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (ISFCP/IAEC/M5/2012/P39), and experiments were conducted in compliance with the guidelines of the Indian National Science Academy (INSA) for the use and care of experimental animals. Cage changes, including food and water, were performed once per week. The animals were randomly divided into 6 groups, and each treatment group comprised of 9 animals (total no of animals = 54). The experimental groups are summarized in Table 1.

Drugs and Pre-treatment Schedule

3-NP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in buffered saline (pH 7.4) and administered intraperitoneally (i.p.) at a dose of 10 mg/kg once a day, for a period of 21 days to induce HD-like symptoms. L-theanine (Himedia Laboratories Pvt. Ltd., Nashik, India) was dissolved in distilled water and administered orally at a dose of 25 and 50 mg/kg once a day, 1 h prior to 3-NP treatment. L-arginine and L-NAME (Sigma Chemicals, St. Louis, MO, USA) were diluted with saline (adjust pH 7.4) and administered intraperitoneally to animals 1 h prior to L-theanine administration. IL-1β, IL-6 and TNF-α ELISA Kits were bought from Krishgen Bio. Sys. Ashley, CA. Behavioral parameters like grip strength, narrow beam, rota-rod, and locomotor activity were assessed on weekly interval. Terminally on the 22nd day, animals were sacrificed, and the striatum was separated and was used to estimate biochemical parameters

Table 1 Experimental groups

Experimental group	Treatment
Group 1	Normal control (Saline + DDW treated, p.o.)
Group 2	3-NP (10 mg/kg, i.p.)
Group 3	L-Theanine (25 mg/kg, p.o) + 3-NP (10 mg/kg, i.p)
Group 4	L-Theanine (50 mg/kg, p.o) + 3-NP (10 mg/kg, i.p)
Group 5	L-NAME (10 mg/kg, p.o) + L-Theanine (25 mg/kg, p.o) + 3-NP (10 mg/kg, i.p)
Group 6	L-Arginine (50 mg/kg, p.o) + L-Theanine (50 mg/kg, p.o) + 3-NP (10 mg/kg, i.p)

(LPO, nitrite, reduced GSH) and neurotransmitters analysis (GABA, glutamate, adenosine, DA, NE, serotonin, DOPAC, HVA,) and levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β).

Measurement of Body Weight

The body weight of animals was recorded just before 3-NP administration (first day) and on the last day of the study (21st day). Percentage change in body weight was calculated using formula:

Change in Body Weight

$$= \text{Body weight } (1^{st}\text{day- } 21^{st}\text{day}) / 1^{st}\text{day body weight}$$

$$\times 100$$

Behavioral Assessments

Rotarod Activity

All animals were evaluated for motor coordination and integrity on rotarod test on week interval. Briefly, the Rotarod apparatus comprises of a rod 30-cm long and 7 cm in diameter and operates at a constant speed of 25 rpm (IMCORP, Ambala, India). Animals were given a prior training session before the initialization of experiment. Animals were placed back on the rotarod after falling off during acclimation sessions. The three measured latencies to fall during experiment were averaged to produce a final value, and cut-off time was 180 s [7].

Open Field Test

Open field test is used to monitor spontaneous locomotor activity using a wooden, rectangular, light brown-colored open field apparatus measuring $100 \times 100 \times 40$ cm. The floor of the apparatus was divided into 25 rectangular squares. The experimental room was illuminated by 40 W white bulb located 150 cm above the test apparatus. Each crossing was considered only when all 4 paws were moved to another square.

Animals were placed in apparatus for 12 min, and total activity of animals (number of squares crossed, rearing, grooming and sniffing) in last 10 min was recorded [6].

Grip Strength Measurement

Grip strength of the fore limbs was measured using digital grip force meter (DFIS series, Chatillon, Greensboro, NC, USA). The rat was positioned to grab the grid with the fore limbs and was gently pulled to record the grip strength [4]. The grip strength was recorded in Kgf.

Beam-Crossing Task

Narrow beam comprised of two platforms (8 cm in diameter) connected by a wooden beam (0.5 mm in thickness, 2.0 cm in width, and 120 cm in length). The beam was elevated 1 m above ground. A box filled with sawdust was placed below the beam, serving as protection for a falling rat. For acclimation, a rat was allowed to explore it for 5 min before training. When a rat walked across the beam from one end to the other end, number of slips and time taken to cross the beam in each trial was recorded. The three measured transfer latencies were averaged to produce a final value and inter-trial interval is 2 min. [7].

Dissection and Homogenization

On the 22nd day, animals from each group were randomly divided into three sub-groups, each consisting of 3 animals, first for biochemical estimations, second for neuroinflammatory markers estimations, and third for neurochemicals estimation. Animals were sacrificed by decapitation; the brains were removed and placed in deep freezer at −80 °C until analysis. On the day of experiment, the brains were removed from deep freezer, and the striatum was separated by putting on ice, weighed and homogenized using 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000g for 15 min, and aliquots of the supernatant was

separated and used for biochemical and neuroinflammatory estimation.

Measurement of Oxidative Stress Parameters

Measurement of Lipid Peroxidation

The quantitative measurement of lipid peroxidation in the brain striatum was performed according to the method of [13]. The amount of malondialdehyde (MDA), a measure of lipid peroxidation, was measured by reaction with thiobarbituric acid at 532 nm using a Shimazdu spectrophotometer.

Estimation of Nitrite

The accumulation of nitrite in the striatum supernatant, an indicator of the production of nitric oxide (NO), was determined by a colorimetric assay with Greiss reagent (0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride, 1 % sulfanilamide, and 2.5 % phosphoric acid) as described by [14].

Estimation of Glutathione Levels

Reduced glutathione (GSH) in the striatum was estimated according to the method described by [15].

Protein Estimation

The protein was measured by the Lowry method using Folin phenol reagent [16].

Estimation of Tumor Necrosis Factor-Alpha (TNF-α) and IL-1β and IL-6 in Striatum

The quantifications of TNF-α and IL-1β were done by rat TNF-α, IL-1β, and IL-6 immunoassay kit (KRISHGEN BioSystem, USA). The quantikine rat TNF-α, IL-1β, and IL-6 immunoassay is a 4.5-h solid-phase ELISA designed to measure rat TNF-α, IL-1β, and IL-6. It is a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) using a microtitre plate reader. Concentrations of TNF-α, IL-1β, and IL-6 were calculated from the standard curves.

Neurochemical Analysis

Estimation of Brain Catecholamines

The estimation of brain catecholamines was done by method described by Jamwal et al. [6]. Concentrations of neurotransmitter and their metabolites were calculated from the standard curve generated by using standard in a concentration range of

10–100 ng/ml. The values are expressed as percentage of normal control group.

Brain GABA and Glutamate Estimation by HPLC-ECD

The estimation of GABA and glutamate was done by method described by Jamwal et al. [6]. Amino acids were measured as OPA/β-ME derivatives according to the method of Donzanti and Yamamoto [17]. Concentrations of amino acids were calculated from the standard curve generated by using standard in a concentration range of 10–100 ng/ml. The values are expressed as percentage of normal control group.

Brain Adenosine Estimation by HPLC-PDA

Adenosine levels were estimated by HPLC using photodiode array detector according to the method described by [18]. Concentrations of neurotransmitter and their metabolites were calculated from the standard curve generated by using standard in a concentration range of 10–100 ng/ml. The values are expressed as percentage of normal control group.

Statistical Analysis

The data obtained is expressed as mean ± S.E.M. The behavioral parameters at different time points were analyzed by repeated measures two-way ANOVA followed by Bonferroni's multiple comparison, and biochemical parameters were analyzed by one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

Results

Effect of L-Theanine on 3-NP-Induced Decrease in Body Weight of Rats

3-NP treatment induces significant loss in body weight as compared to normal control group ($p < 0.001$) on the last day (21st day). Pretreatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly and dose dependently prevented the alteration in body weight as compared to the 3-NP-treated groups ($p < 0.05$) (Fig. 1). Concurrent pre-treatment of L-NAME with L-theanine (25 mg/kg/day, p.o) significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$).
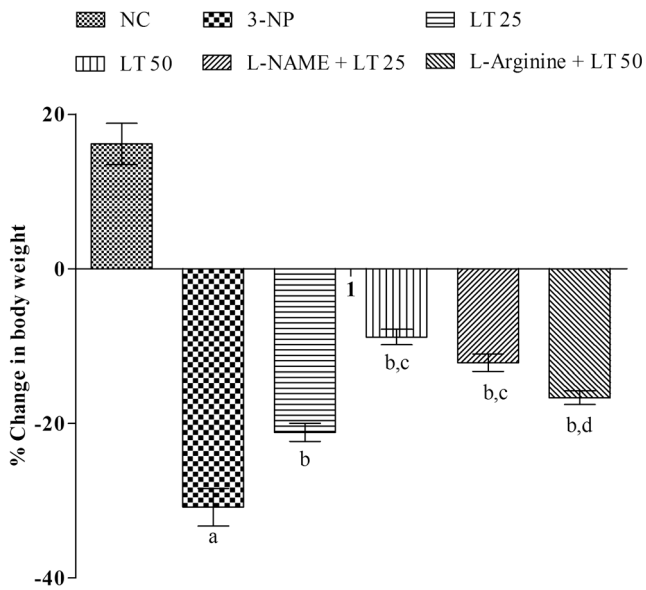
Fig. 1 Effect of L-theanine on body weight in 3-NP-treated rats ($n = 9$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by one way ANOVA followed by Tukey's post hoc test. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50*, L-theanine (50 mg/kg, p.o.)

Fig. 2 Effect of L-theanine on rotarod activity in 3-NP treated rats ($n = 9$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by repeated measures two way ANOVA followed by Bonferroni's multiple comparison. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Effect of L-Theanine on 3-NP-Induced Changes 3-NP-Induced Changes in Locomotor Activity, Rotarod, and Grip Strength Performance of Rats

Systemic administration of 3-NP significantly administration significantly decreased grip strength (on rotarod and grip strength meter) and locomotor activity (day 14th and 21st) when compared to normal control group ($p < 0.001$). Pre-treatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly and dose dependently ameliorated the impairment in grip strength and locomotor activity as compared to 3-NP group ($p < 0.05$) (Figs. 2, 3, and 4). Concurrent pre-treatment of L-NANE with L-theanine (25 mg/kg/day, p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$)

Effect of L-Theanine on Narrow Beam Walks Parameter in 3-NP-Treated Rats

3-NP administration significantly increased the transfer latency and foot errors on narrow beam walk on day 21st as compared to normal control group ($p < 0.001$) (Fig. 5). Pretreatment with L-theanine (5 and 10 mg/kg/day, p.o) significantly and dose dependently
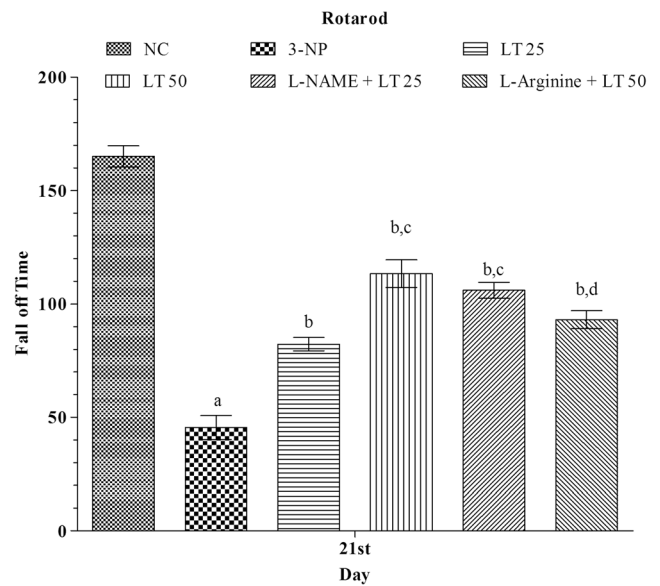
prevented the increase in latency and foot errors on narrow beam walk apparatus as compared to 3-NP treated rats ($p < 0.05$). Concurrent pre-treatment of L-NAME
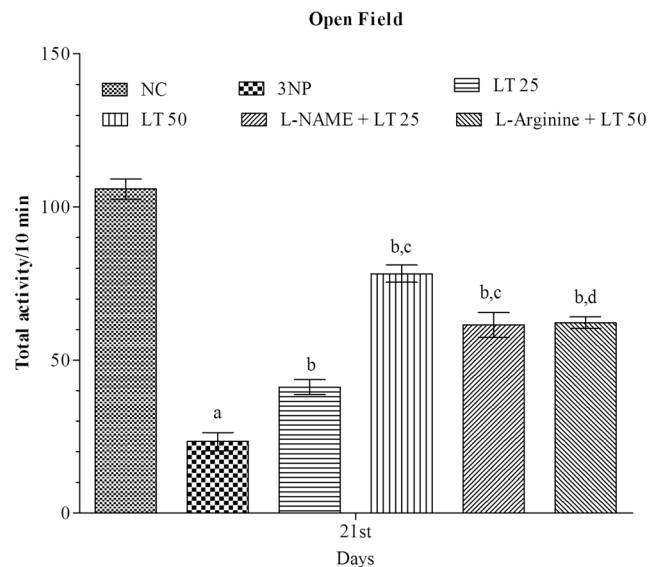

Fig. 3 Effect of L-theanine on locomotor activity in 3-NP-treated rats ($n = 9$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by repeated measures two way ANOVA followed by Bonferroni's multiple comparison. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal Control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)
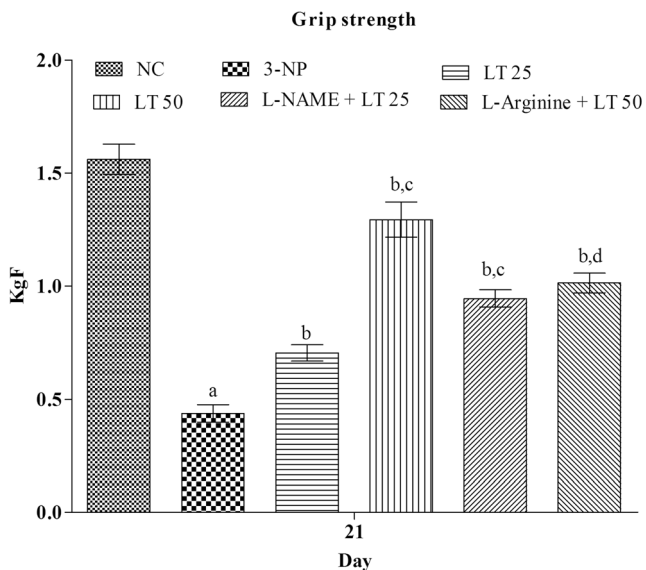
Grip strength

Fig. 4 Effect of L-theanine on grip strength in 3-NP-treated rats ($n = 9$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by repeated measures two-way ANOVA followed by Bonferroni's multiple comparison. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal Control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

with L-theanine (25 mg/kg/day, p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$).
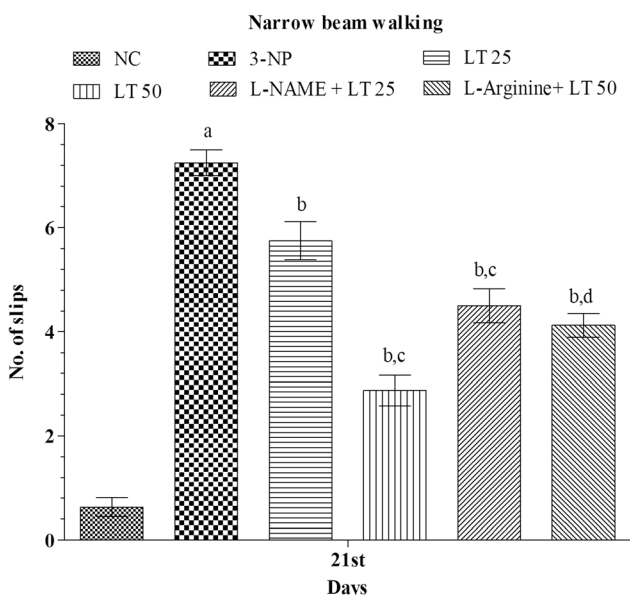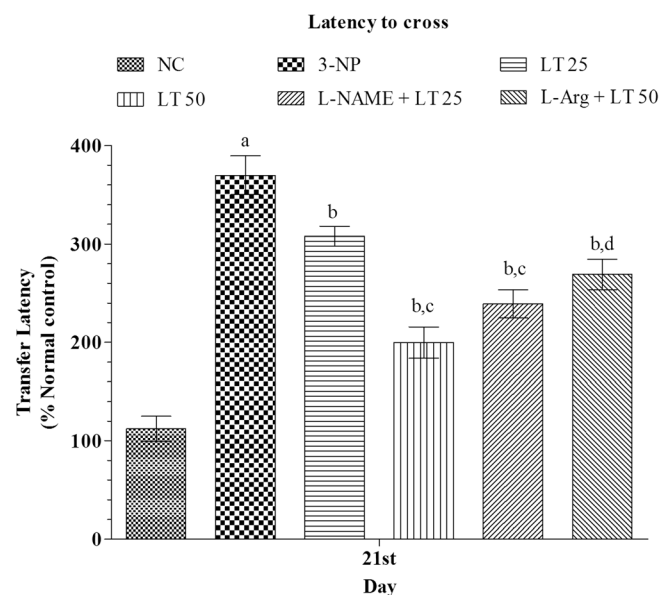
Effect of L-Theanine on 3-NP-Induced Oxidative Stress in Rats Striatum

Systemic administration of 3-NP significantly increased oxido-nitrosative stress parameters, i.e., MDA and nitrite level in the striatum were significantly higher and reduced GSH level was significantly lower in 3-NP group as compared to the normal control group ($p < 0.001$) (Table 2). Pre-treatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly and dose dependently prevented the increase in oxido-nitrosative stress in 3-NP administered rats ($p < 0.05$). Concurrent pre-treatment of L-NAME with L-theanine (25 mg/kg/day, p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$).

Effect of L-Theanine on TNF-α, IL-6, and IL-1β Levels in the Striatum of 3-NP-Treated Rats

Systemic administration of 3-NP significantly increased the level of pro-inflammatory cytokines, i.e., TNF-α, IL-6, and IL-1β levels in the striatum as compared to normal control group ($p < 0.001$) (Fig. 6). Pre-treatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly and dose dependently prevented the increase in levels of TNF-α, IL-6, and IL-1β as compared to the 3-NP alone-treated groups ($p < 0.05$). Concurrent pre-treatment of L-NAME with L-theanine (25 mg/kg/day,

Narrow beam walking

Fig. 5 Effect of L-theanine on narrow beam walking in 3-NP-treated rats ($n = 9$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by repeated measures two-way ANOVA followed by Bonferroni's multiple comparison. *a* $p < 0.01$ versus NC, *b* $p < 0.05$

Latency to cross

versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal Control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Table 2 Effect of L-theanine on oxidative stress in 3-NP-treated rats ($n = 3$). Values are expressed as mean ± SEM. Data was analyzed by one-way ANOVA followed by Tukey's post hoc test

Treatment group	MDA (nmol/mg protein)	Nitrite level (μg/ml)	GSH (μmol GSH/mg protein)
NC 165 ± 12 154 ± 1	2.15 ± 0.52	119 ± 9.85	0.110 ± 0.019
3-NP	7.85 ± 0.62[a]	245 ± 10.57[a]	0.029 ± 0.005[a]
LT 25	5.89 ± 0.54[b]	210 ± 4.65[b]	0.048 ± 0.009[b]
LT 50	4.15 ± 0.54[b, c]	170 ± 5.80[b, c]	0.074 ± 0.011[b, c]
L-NAME + LT 25	4.35 ± 0.40[b,c]	185 ± 6.75[b, c]	0.066 ± 0.008 [b,c]
L-Arginine + LT 50	5.10 ± 0.35[b, d]	198 ± 6.75[b, d]	0.058 ± 0.006[b, d]

NC normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

[a] $p < 0.01$ versus NC

[b] $p < 0.05$ versus 3-NP

[c] $p < 0.05$ versus LT 25

[d] $p < 0.05$ versus LT 50

p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$).

Effect of L-Theanine on Striatal NE, DA, 5-HT, DOPAC, and HVA Levels

NE, DA, and 5-HT were found to be significantly decreased in 3-NP-treated rats as compared to normal control group ($p < 0.001$) whereas pretreatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly prevented this decrease in NE, DA, and 5-HT levels in the striatum ($p < 0.05$). Further, treatment with 3-NP significantly enhance DOPAC and HVA levels in striatum ($p < 0.001$). Pre-treatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly prevented the alteration in levels of NE, DA, 5-HT, DOPAC, and HVA levels compared to 3-NP alone group ($p < 0.05$). Concurrently, the DA turnover was found to be elevated upon 3-NP treatment ($p < 0.001$) and pre-treatment with L-theanine (5 and 10 mg/kg/day, p.o) lowered it significantly as compared to 3-NP ($p < 0.05$). Concurrent pre-treatment of L-NAME with L-theanine (25 mg/kg/day, p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$) (Figs. 7, 8, and 9).
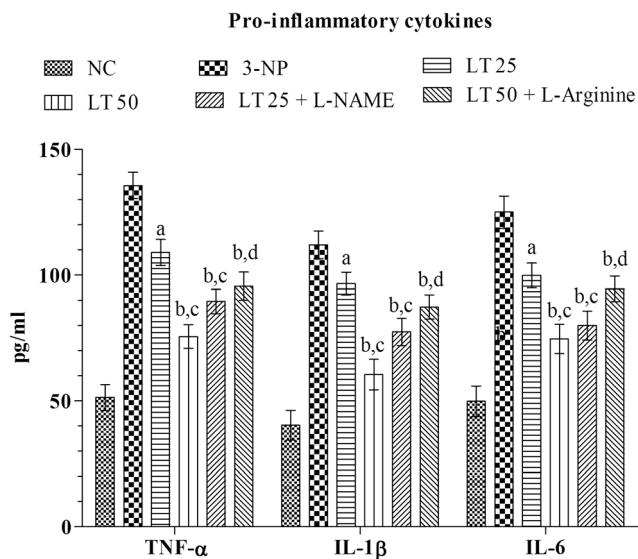
Effect of L-Theanine on Striatal GABA and Glutamate Levels

3-NP treatment significantly lowered GABA level and enhanced glutamate level in 3-NP alone-treated group when compared to normal control group ($p < 0.001$). Pre-treatment with L-theanine (25 and 50 mg/kg/day, p.o) significantly and dose dependently prevented the alteration in GABA and glutamate level in striatum ($p < 0.05$). Concurrent pre-treatment of L-NAME with L-theanine (25 mg/kg/day, p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$). (Fig. 10)
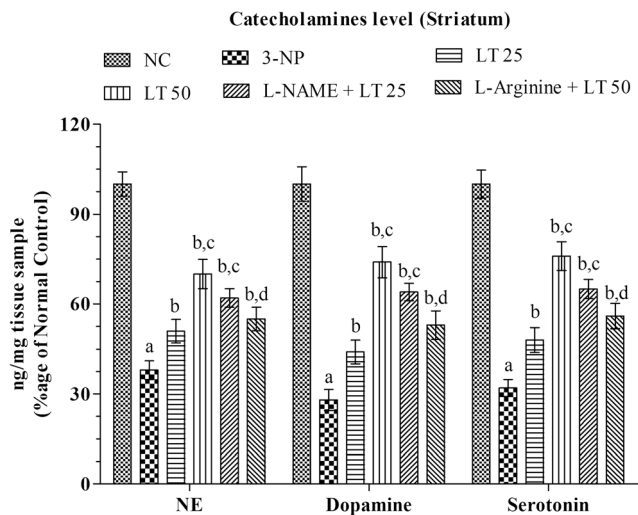

Fig. 6 Effect of L-theanine on TNF-α, IL-1β, and IL-6 levels in 3-NP-treated rats ($n = 3$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Fig. 7 Effect of L-theanine on striatal catecholamines levels in 3-NP-treated rats ($n = 3$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Fig. 9 Effect of L-theanine on striatal dopamine turnover in striatum in 3-NP-treated rats ($n = 3$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Effect of L-Theanine on Striatal Adenosine Levels

Systemic administration of 3-NP significantly ($p < 0.001$) decreased the level of adenosine in the striatum as compared to normal control group. Pre-treatment with L-theanine (5 and 10 mg/kg/day orally) for 21 days significantly and dose depen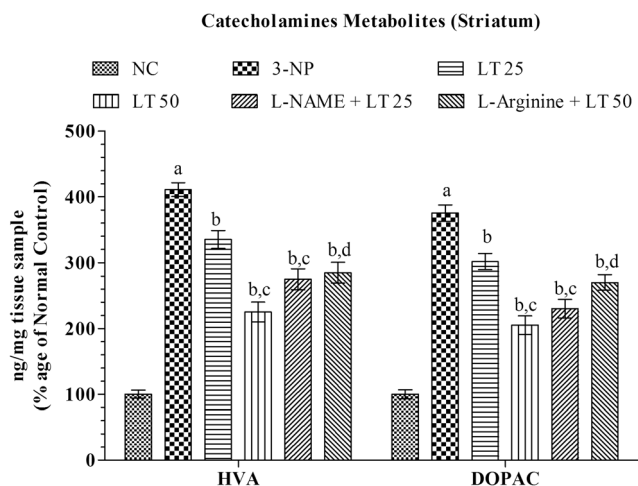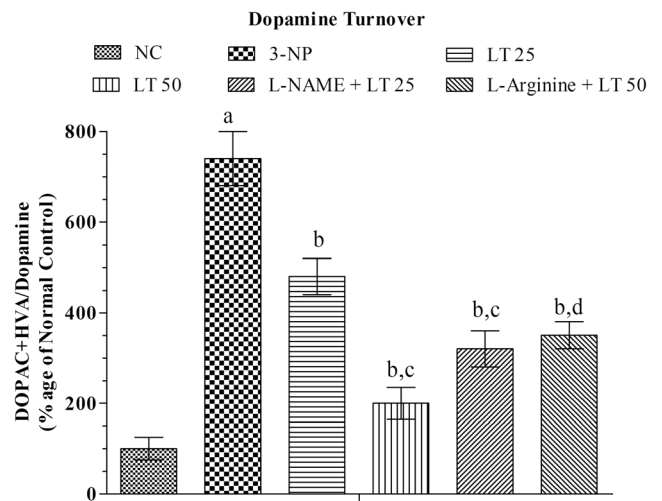dently prevented alteration in the levels of adenosine ($p < 0.05$) (Fig. 10). Concurrent pre-treatment of L-NAME with L-theanine (25 mg/kg/day, p.o) and L-NAME significantly enhanced the protective effect of L-theanine (25 mg/kg, p.o) ($p < 0.01$) whereas pre-treatment of L-arginine with L-theanine (50 mg/kg/day, p.o) significantly decreased the protective effect of L-theanine (50 mg/kg, p.o) ($p < 0.01$).
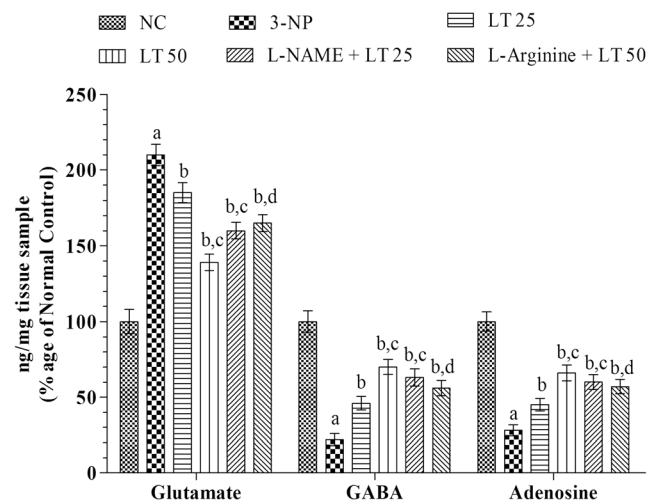

Fig. 8 Effect of L-theanine on striatal catecholamines metabolite level in 3-NP-treated rats ($n = 3$). *Data columns* represent mean of values, and *error bars* represent SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Fig. 10 Effect of L-theanine on striatal GABA, glutamate and adenosine levels in 3-NP-treated rats ($n = 3$). *Columns* represent mean of values, and *error bars* represent SEM. Data analyzed by one-way ANOVA followed by Tukey's post hoc test. *a* $p < 0.01$ versus NC, *b* $p < 0.05$ versus 3-NP, *c* $p < 0.05$ versus LT 25, *d* $p < 0.05$ versus LT 50. *NC* normal control, *3-NP* 3-nitropropionic acid (10 mg/kg, i.p.), *LT 25* L-theanine (25 mg/kg, p.o.), *LT 50* L-theanine (50 mg/kg, p.o.)

Discussion

The present study has revealed the neuroprotective profile of L-theanine against 3-NP-induced neurotoxicity in rats. Our study confirms the involvement of nitric oxide (NO) pathway in 3-NP-mediated neurotoxicity and provides indication that NO modulation is involved in the protective effect of L-theanine. Treatment with L-theanine significantly and dose dependently prevented 3-NP-induced motor-deficit, oxidative stress, proinflammatory cytokines levels and restored striatal GABA, glutamate and catecholamine levels in rats.

Systemic administration of the mitochondrial toxin 3-NP to rats induces a spectrum of HD-like neuropathology in the rat striatum and serves as an good experimental model of HD [3, 4, 7]. 3-NP model mimics and reproduces the hyperkinetic and hypokinetic symptoms of HD, depending on the time and dose administered, thus allowing the initial (or early) and late phases of HD to be evaluated [12]. Chronic administration of 3-NP at low doses (10 mg/kg/day, 3–6 weeks) induces sustained state of metabolic alterations and some other features similar to those displayed by HD patients [12]. In the present study, 3-NP (10 mg/kg/day for 3 weeks) treatment produces stable motor-deficit as evidenced by increase in transfer latency in narrow beam walk, loss of grip strength, decrease in fall of time in rotarod test, indicating motor impairment and striatal dysfunction. Our results stay in good agreement with previous studies demonstrating similar movement disabilities following 3-NP administration [4, 7, 19]. In addition, increased production of NO, oxidative stress, and sensitization of NMDA receptor play a central role in 3-NP-induced striatal toxicity in both rodents and primates [3]. 3-NP also induces the release of NO through stimulation of NOS activity. NO reacts with O_2 to produce $ONOO^-$, which is highly cytotoxic and has capability to induce both protein nitration and hydroxyl radical (^-OH) formation [12].

A close relationship exists between impairment in motor function and disruption of neurotransmitters homeostasis in basal ganglia in HD. Dysregulated neurotransmitter signaling is well implicated in HD patients and as well as in experimental animals [6, 8, 19]. In queue with previous studies, our study also confirms motor deficiency and disruption of neurotransmitters homeostasis and pre-treatment with L-theanine prevented these alterations. Also, pretreatment of L-arginine and L-NAME with L-theanine significantly reversed and potentiated the protective effect of L-theanine, respectively. Our study results clearly depict the involvement of NO pathway in protective effect of L-theanine. Recently, one study claims that L-theanine possesses neuroprotective potential and improved motor performance in 3-NP model of HD [20].

Numerous pieces of evidences show altered levels of catecholamines and their metabolites in HD animals, patients, and postmortem brains. 3-NP treatment causes excessive release of glutamate and subsequent sensitization of NMDA receptor

[12, 21]. A recent study from our lab suggest that 3-NP treatment produces decline in NE, DA, and 5-HT levels, enhances glutamatergic signaling and decreases GABAergic signaling [19]. In the present study, systemic administration of 3-NP significantly decreases GABA, DA, NE, 5-HT and increases glutamate level. These results are in good agreement with earlier observation [19]. 3-NP-induced alteration in DA level has been reported to disrupt the delicate balance of neurotransmitters, e.g., NE, 5-HT, acetylcholine, GABA, and glutamate, especially within basal ganglia circuit [8], which can be well correlated with observed motor impairment in the present study. Previous studies have reported that L-theanine administration increase brain DA, NE and 5-HT, GABA levels and inhibits glutamate-mediated excitotoxicity [22]. Thus, improvement of striatal neurotransmitters profile and NO modulation mechanism may contribute in a part to the observed beneficial effects against 3-NP-induced motor deficit.

Adenosine performs dual function as a neurotransmitter and neuromodulator in the central nervous system. Adenosine has been reported to release in response to excessive glutamate release and it acts pre-synaptically to inhibit the release of glutamate and post-synaptically to inhibit the excitatory actions of glutamate [23–26]. In the present study, systemic administration of 3-NP significantly decreases the adenosine level in the striatum whereas L-theanine pre-treatment significantly prevented the decrease in adenosine and its metabolites level in striatum. The protective effect of L-theanine may be due to normalization of striatal neurotransmitters level, anti-inflammatory, and antioxidant property, which spares the neurons from detrimental effect of 3-NP.

Oxidative stress and excitotoxicity are well reported to occur after the inhibition of complex-II in 3-NP treated animals [3, 4, 27]. Mitochondrial complex-II inhibition leads to massive Ca^{2+} influx mainly through voltage-gated membrane channels and voltage-gated NMDA receptor-channel complex which further leads to activation of nitric oxide synthase (NOS) via Ca^{2+}/calmodulin and the subsequent production of NO^- [3]. In support of the above mentioned reports, in the present study, 3-NP treatment significantly inhibited complex II and enhanced oxido-nitrosative stress and level of proinflammatory cytokines levels in the striatum. Our results are consistent with previous reported studies [4, 19]. In the present study, pre-treatment with L-theanine significantly attenuated the abnormal levels of all biochemical and neuroinflammatory parameters in 3-NP rats. These results are in good agreement with previous reports [20]. Also, pretreatment of L-arginine with L-theanine significantly increased the oxido-nitrosative stress and neuroinflammation whereas pretreatment of L-NAME with L-theanine significantly inhibited the oxido-nitrosative stress and neuroinflammation. The results of our study are suggestive

of L-theanine antioxidant and anti-inflammatory properties, which could be mediated in part via inhibition of NO production in striatum.

L-theanine displays wide variety of neuroprotective action due to structural similarity with GABA and glutamate. L-theanine is suggested to act as agonist for GABA receptor and thereby increasing GABA level. In particular, L-theanine directly provides neuroprotection against focal cerebral ischemia, excitotoxic cell death induced by kainic acid and glutamate [22, 28]. Neurochemistry studies suggested that L-theanine increases the density and level of brain NE, dopamine, 5-HT, GABA, possess micromolar affinity for AMPA and NMDA receptor, promotes the formation of nerve growth factors like BDNF and GDNF, and accelerates the development of the central nervous system [29, 30]. It has been reported that neuroprotective effect of L-theanine is mediated, at least in part by $GABA_A$ receptor and glutamate transporters. L-theanine is also reported to induce reduction in glutamate reuptake by inhibition of glutamate transporter [22]. L-theanine inhibits activation of c-Jun N-terminal kinase and caspase-3 induced by L-glutamate [31]. Also, L-theanine decreased the production of NO induced by glutamate by downregulation of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) protein activity [31]. In addition to this, L-theanine has been reported to boost locomotor activity in *Drosophila* male flies [32]. Recently, L-theanine has been reported to restore normal architecture of brain regions and downregulates the expression of inflammatory cytokines against PCBs (Aroclor 1254)-induced oxidative damage in the rat brain [33]. Also, L-theanine pre-treatment provides neuroprotection against aluminum induced neurotoxicity in the cerebral cortex, hippocampus, and cerebellum of the rat brain [34]. Nonetheless, for the first time, our result demonstrates that L-theanine-mediated neuroprotection involves NO pathway modulation against 3-NP-induced neurotoxicity.

Conclusion

The data obtained suggests that high dose administration of L-theanine (50 mg/kg) produced pronounced effect in preventing the development of 3-NP induced neurotoxicity. The neuroprotective effect of L-theanine is attributed to preservation of striatal neurotransmitters homeostasis, free radical scavenging, antioxidant activity, and anti-inflammatory potential, which spares the neurons from death. This suggests that L-theanine may be a better preventive strategy to provide optimal neuroprotection against development of 3-NP-induced neurotoxicity. Further studies are warranted before approaching to any final implications.

Compliance with Ethical Standards   The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (ISFCP/IAEC/M5/2012/P39), and experiments were conducted in compliance with the guidelines of the Indian National Science Academy (INSA) for the use and care of experimental animals.

Conflict of Interest   The authors declare that they have no conflict of interests.

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