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Treatment with *Tinospora cordifolia* ameliorates prenatal stress and maternal separation-induced memory deficits by restoring amygdaloid neuronal architecture

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ABSTRACT

Introduction: *Tinospora cordifolia* (TC) possesses antioxidative properties and has been shown to improve cognition. In this study, the effects of TC were investigated on prenatal vibratory stress, maternal separation-induced amygdala plasticity, and memory retention in pregnant rats and their neonates.

Methods: Four experimental animal groups of pregnant Wistar rats were employed in this research, including normal & vehicle controls, the stressed group, which received 3 hours of vibratory stress/day, and the TC-treated group, which received 6 mg/kg TC before vibratory stress. The neonates born to these mothers were then subjected to maternal separation, followed by postnatal TC treatment. After 6 weeks, the animals were assessed to evaluate memory retention, serum cortisol levels, and neuronal structural changes in the amygdala.

Results: Neonates exposed to prenatal vibratory stress and maternal separation entered the dark compartment more quickly during the retention test ($P<0.001$), indicating reduced memory retention. In contrast, the TC-treated groups showed longer latencies ($P<0.001$), suggesting improved memory retention. The TC-treated mothers and neonates had longer dendrites with more branching points and intersections ($P<0.001$). However, these dendrites underwent pruning, indicating a complex structural response to stress and TC treatment.

Conclusion: The results indicate that TC exerts neuroprotective effects against prenatal vibratory stress and maternal separation and aids memory retrieval in rats by restoring amygdala neuronal damage.

Implication for health policy/practice/research/medical education:
The preclinical research findings demonstrate the memory-enhancing effects of *Tinospora cordifolia* extract. Hence, this extract may be useful during pregnancy to support fetal neurogenesis and protect against neuronal damage caused by prenatal stress and postnatal maternal separation in humans.

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Introduction

Early-life stress is a significant risk factor for psychiatric ailments such as major depressive disorder and anxiety. Prenatal stress and maternal isolation affect brain development and alter structural and functional connectivity in offspring. During pregnancy, neuronal proliferation, synaptic connections, and pruning might be disrupted by maternal stress (1). Stress during early gestation is known to cause hypothalamus-pituitary-adrenal (HPA) axis dysregulation and an increase in

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maternal glucocorticoids, which cross the placental barrier and influence neurodevelopmental trajectories (2). The stress hormone, cortisol, plays a key role in the development of limbic regions of the brain but abnormally elevates the level of maternal cortisol during pregnancy, resulting in neurotoxicity and reduced fetal brain growth (2,3).

The Amygdala, a subcortical structure that is part of the limbic lobe of the brain, is responsible for emotional processing, fear conditioning, and stress management (4). The amygdala influences many cognitive processes, including memory, attention, and decision-making (5). It is one of the fundamental parts of the neural circuitry controlling sociosexual and defensive behaviors (6). Earlier research in rodents and primates revealed that prenatal stress exposure during the early developmental period has adverse consequences on the developing amygdala and hippocampus (2,3,7).

Tinospora cordifolia (TC) is a widely used herb in folk and Ayurvedic systems of medicine (8). The ethnopharmacological data from earlier studies suggest that TC has a wide range of active phyto-compounds that have antistress, antidiabetic (9,10), antioxidation (11,12), anticancer (13), antimicrobial, and immunomodulator properties (14-18). The existing data are insufficient to confirm the neuroprotective effect of TC on neuronal damage resulting from prenatal vibratory stress and maternal separation. Hence, the current study employed a novel vibratory stress model to evaluate TC's potential to mitigate amygdaloid neuronal damage and enhance memory retention in newborn rats exposed to these stressors. The findings can contribute valuable insights into various prenatal stress models and the antistress properties of TC specific to these stressors.

Materials and Methods

Experimental animals

Female albino Wistar rats bred in the central animal house of our university were used in the study. The animals were maintained under a 12-hour light/dark cycle at a controlled temperature and humidity. The rats were fed standard food pellets and water *ad libitum*. Approval was obtained from the Institutional Animal Ethical Committee (Reference No. IAEC/KMC/56/2015) before the commencement of the experiment.

Study groups

The experiment consisted of four groups of pregnant rats, namely, normal control mothers (NC_M), vehicle control mothers (VC_M), vibratory stressed mothers (VS_M), and vibratory stress + TC treatment mothers (VS+TC_M). Each group consisted of 6 animals.

The NC_M mothers remained in their home cages undisturbed, while the VC_M rats received 5% saline (an equal volume of TC treatment). The VS_M mothers were exposed to 3 hours of vibratory stress every day from gestational days 7-16. The duration of vibratory stress was standardized during our pilot study. VS+TC_M group animals were treated with 6 mg/kg body weight TC extract before they were subjected to 3 hours of vibratory stress from gestational days 7-16.

The neonates born to the control group were divided into normal control neonates (NC_N, n=6) and vehicle control neonates (VC_N, n=6). The neonates born to mothers with vibratory stress were further divided randomly into vibratory stressed control neonates (VS_N Control, n=8), vibratory stressed neonates subjected to maternal separation for 6 hours from postnatal days 1-7 (VS+MS_N, n=8), and vibratory stressed neonates treated with 6 mg/kg/body weight of TC from postnatal days 1-7 (VS+PNTC_N, n=8). The neonates born to vibratory-stressed and TC-treated mothers were divided randomly into vibratory-stressed and TC-treated neonates (VS+TC_N Control, n=8), vibratory-stressed and TC-treated neonates subjected to maternal separation for 6 hours from postnatal days 1-7 (VS+TC+MS_N, n=8), and vibratory stressed and TC-treated neonates subjected to maternal separation for 6 hours along with 6 mg/kg body weight of TC treatment from postnatal days 1-7 (VS+TC+MS+PNTC_N, n=8).

Induction of vibratory stress

An indigenously developed vibratory machine was used to induce vibratory stress in pregnant rats. Animals were maintained in their cages on the vibratory machines. To simulate the vibrations in a moving vehicle, the machine was built to produce horizontal and vertical oscillations. It was set to produce 72 oscillations per minute after the preliminary experiments. The process, intensity, and duration of vibratory stress induction were previously standardized.

The pregnant animals were kept on the horizontal oscillatory tray of the machine to induce the stress from their gestational day 7 to 16 every day for 3 hours. Following the vibratory stress induction, the daily observations of altered eating patterns and behaviors were noted.

Induction of postnatal maternal separation stress

Neonates born to prenatally stressed dams and neonates born to prenatally stressed + TC treated dams were separated from their mothers from their postnatal day 1 (PND1) to 7 (PND7) for 6 hours every day to induce maternal separation stress. Neonates were returned to their respective mothers after 6 hrs for weaning. Every day, the changes in the body weight, tail length, and eating habits of these newborns were noted.

Administration of *Tinospora cordifolia*

The pure extract of TC (with shelf life of 2 years) was procured from the herbal manufacturer (Himalaya Wellness Company, Bengaluru, India). The pure TC extract of 6 mg/kg body weight was administered orally to

dams of drug treatment groups along with 5% saline as the vehicle, using an oral feeding tube and syringe every day before subjecting them to vibratory stress. The neonates of postnatal TC treatment groups also received 6 mg/kg body weight of TC pure extract. The dosage of the TC extract for treatment groups was standardized during the preliminary studies.

Passive avoidance test to assess memory retention power (19)

a. Apparatus

A locally designed and fabricated passive avoidance apparatus with a larger compartment (50×50×35 cm) and a smaller compartment (15×15×15 cm) was used in the study. Both compartments had metal grid floors, wooden walls, and roofs that could be opened or closed. The larger brightly illuminated compartment was connected to the smaller dark compartment, and an opening measuring 6×6 cm was made. The metal grid of the smaller compartment was electrifiable because it was connected to a current stimulator.

b. Procedure

The experiment included three parts: exploration, aversive stimulation, and learning (passive avoidance acquisition), with a retention test.

Exploration

There were 3 trials of exploration. During each trial, the animal was placed in the illuminated larger compartment facing its tail end toward the entrance of the smaller compartment. Communication between the larger and smaller compartments was left open for 3 minutes, and the animal was allowed to explore the apparatus. The latency to enter the smaller compartment and the time spent by the rat in the smaller compartment were recorded for every trial. Following each trial, an intertrial interval of 5 minutes was given during which the rat was kept in the home cage.

Passive avoidance acquisition

After 3 trials of exploration, the animal was kept in the closed smaller compartment and three strong electric foot shocks of 50 Hz and 1.5 mA were administered for a second. Between two shocks, an interval of five seconds was given. After the administration of foot shock, the animals were returned to their home cages.

Passive avoidance retention test

Twenty-four hours after the passive avoidance acquisition, the first retention test was conducted. The second retention test was performed on the 6th day after the acquisition. The procedure for the retention test was similar to that for the passive avoidance acquisition test, as explained earlier. During each trial, the latency to enter the smaller compartment and the total time spent by the rat in the small compartment were recorded.

Biochemical estimation of serum cortisol

Blood was extracted retro-orbitally and collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes from the animals of each group before they were sacrificed. The serum was separated using a centrifuge and used for the estimation of cortisol.

Brain tissue collection

After the completion of the experiment, the mothers and pups were sacrificed. The brains were removed from the cranial cavity; the coronal sections of each cerebral hemisphere were taken and processed for Golgi cox staining.

Golgi-Cox staining procedure

Golgi-Cox solution preparation

The equivolumes of mercuric chloride and potassium chromate were mixed with the same volume of potassium dichromate using a magnetic stirrer under a fume hood and kept in the dark for two days.

Golgi-Cox stain impregnation

Freshly dissected brain pieces were kept in Golgi-Cox solution for one week after rinsing in double distilled water. Later, they were kept in 30% sucrose solution for 8 hours, and 120 µm thick sections were cut using a sledge microtome. Later, the sections were treated with 75% ammonia solution and 1% sodium thiosulfate for 10 minutes in the dark to fix the stain and processed with different grades of alcohol for dehydration.

Microscopic observation of amygdaloid neurons for dendritic quantification

Following Golgi-Cox staining, a series of photographs were taken using a Moticam 580 5.0 MP microscope at 20X magnification to study the structure of amygdaloid neurons. Dendritic arborization of the neuronal amygdala was quantified using Sholl's circle method (20,21).

Statistical analysis

The data obtained were analyzed using Statistical Package for Social Sciences (SPSS) version 20. The results were presented as mean ± standard error of the mean (SEM). To compare the mean values of different groups, we employed a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A significance level of $P<0.05$ was considered statistically significant.

We also calculated Pearson's correlation coefficient (r) to analyze the relationship between serum cortisol levels and the latency of animals to enter the dark compartment during the passive avoidance test. This correlation analysis helped us understand any potential associations between stress hormone levels and memory retention abilities in our experimental animals.

Results

Results of the passive avoidance test for assessing memory retention power in mothers

Exploration test

The control, vibratory stressed (VS_M), and TC-treated (VS+TC_M) group animals showed no significant difference in the latency evaluation to enter the dark compartment. Figure 1 shows that the VS_M and VS+TC_M groups spent considerably less time ($P<0.001$) in the dark (small) compartment than the control groups, suggesting that vibratory stress may have caused memory deficits in these animals.

Retention test on the 2nd day: The mean latency time to enter the dark compartment was significantly ($P<0.001$) lower in stressed mothers (VS_M) than in the control and TC-treated mothers during the retention test (Figure 1). As shown in Figure 1, stressed mothers spent significantly ($P<0.001$) more time in the dark (smaller) compartment than did the control and TC-treated mothers (VS+TC_M). A decreased latency to enter the dark compartment and increased time spent in the dark compartment in the VS_M group indicated impaired memory retention.

Retention test on the 6th day: Memory impairment in the VS_M group was evident, as the latency of this test was similar to the latency (in seconds) to enter the dark compartment after 24 hours. Additionally, the stressed mothers (VS_Ms) spent more time in the dark compartment where they received an aversive stimulus, which was associated with compromised memory retention power due to the prolonged effect of vibratory stress (Figure 2).

Results of the passive avoidance test for assessing memory retention power in neonates

Exploration test

During the exploration test, both prenatally stressed (VS_N control) and double stressed (VS+MS_N) neonates displayed a substantial ($P<0.001$) increase in latency to enter the dark compartment and spent more time in the light compartment.

Like the neonates in the control group, the neonates in the TC treatment group entered the dark compartment faster ($P<0.001$) than those in the prenatally stressed and maternal separation groups (Figure 3). Neonates treated with postnatal TC (VS+PNTC_N) demonstrated a significantly shorter latency to enter the dark compartment than the NC_N ($P<0.001$), VS_N control ($P<0.01$), and VS+TC_N control ($P<0.05$) groups. The latency enhancement to enter the dark compartment by VS_N control and VS+MS_N groups indicated anxiety-induced behavioral changes during the exploration test.

Retention test on the 2nd day: Both prenatal and postnatal TC-treated neonates (VS+TC_N control and VS+PNTC_N) had significantly ($P<0.001$) longer latency times to enter the dark compartment than NC_N- and VS_N control-treated neonates, suggesting the retention and retrieval of encoded memories in these TC-treated animals. The fact that prenatally stressed (VS_N control) and double stressed (VS+MS_N) neonates spent significantly ($P<0.001$) more time in the dark compartment than did normal control and TC-treated neonates suggests that prenatal vibratory stress and postnatal maternal separation have an impact on the memory retention power of these neonates.

Retention test after 5 days (Day 6): Both prenatal and postnatal TC-treated neonates (VS+TC_N control and VS+PNT_N) showed persistently longer latency times to enter the dark compartment even 5 days after acquisition, indicating longer retention power and memory retrieval capacity. However, they did not show retention power as did the normal controls. TC-treated neonates also showed a significant ($P<0.001$) decrease in total time spent in the dark compartment compared to prenatally stressed

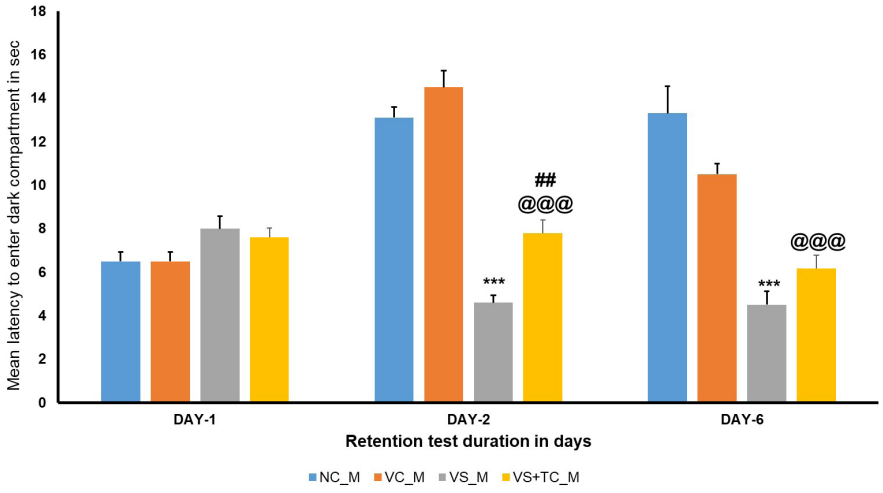

Figure 1. Results of the passive avoidance test (Mean±SEM) of mothers. NC_M: normal control mothers; VC_M: vehicle control mothers; VS_M: vibratory stressed mothers; VS+TC_M: vibratory stressed mothers with *Tinospora cordifolia* treatment. *** $P<0.001$ NC_M vs VS_M; ## $P<0.01$ VS_M vs VS+TC_M; @@@ $P<0.001$ NC_M vs VS+TC_M (Bonferroni post hoc correction).

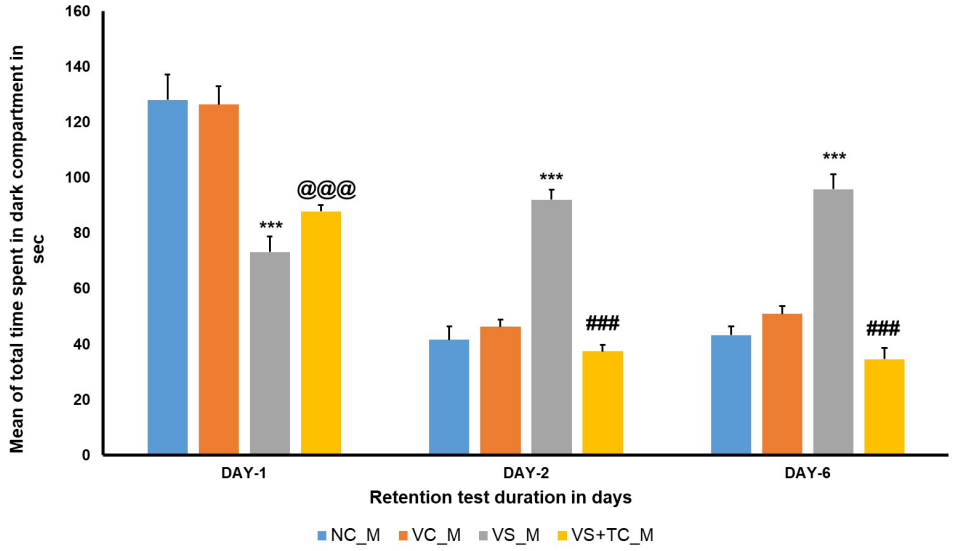

Figure 2. Results of the passive avoidance test (Mean±SEM) of mothers. NC_M: normal control mothers; VC_M: vehicle control mothers; VS_M: vibratory stressed mothers; VS+TC_M: vibratory stressed mothers with *Tinospora cordifolia* treatment. *** $P<0.001$ NC_M vs VS_M; ### $P<0.001$ VS_M vs VS+TC_M; @@@ $P<0.001$ NC_M vs VS+TC_M (Bonferroni post hoc correction).

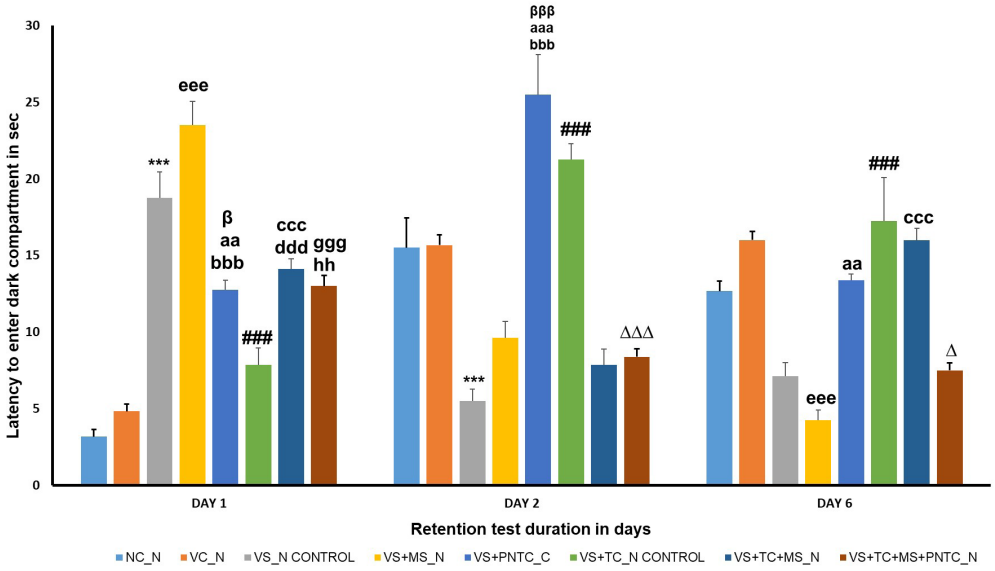

Figure 3. Results of the passive avoidance test of neonates' latency to enter the dark compartment (Mean±SEM). NC_N: normal control neonates, VC_N: vehicle control neonates, VS_N control: prenatally vibratory stressed control neonates, VS+MS_N: prenatally vibratory stressed neonates subjected to maternal separation, VS+PNTC_N: prenatally vibratory stressed neonates subjected to postnatal *Tinospora cordifolia* (TC) treatment, VS+TC_N: prenatally vibratory stressed and TC-treated neonate controls, VS+TC+MS_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and VS+TC+MS+PNTC_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and postnatal TC treatment. *** $P<0.001$ NC_N vs VS_N; ### $P<0.001$ VS_N control vs VS+TC_N control; β $P<0.05$ and βββ $P<0.001$ VS+TC_N control vs VS+PNTC_N; Δ $P<0.05$ and ΔΔΔ $P<0.001$ VS+PNTC_N vs VS+TC+MS+TC_N; aa $P<0.01$ and aaa $P<0.001$ VS_N control vs VS+PNTC_N; bbb $P<0.001$ NC_N vs VS+PNTC_N; ccc $P<0.001$ VS+MS_N vs VS+TC+MS_N; ggg $P<0.001$ VS+MS_N vs VS+TC+MS+PNTC_N; hh $P<0.01$, hhh $P<0.001$ VS_N control vs VS+TC+MS+PNTC_N.

(VS_N control) and double stressed (VS+MS_N) neonates (Figure 4).

Estimation of serum cortisol

The results of serum cortisol estimation in mothers

Both the vibratory stressed (VS_M) and stressed plus TC-treated (VS+TC_M) groups showed significantly increased ($P<0.001$) serum cortisol levels compared to those of the control group animals. However, the serum cortisol levels of the VS+TC_M group animals were relatively low ($P<0.01$) compared to those of the VS_M group animals (Figure 5).

Results of serum cortisol estimation in neonates

Due to prenatal vibratory stress and maternal separation, there was a significantly ($P<0.001$) greater level of serum

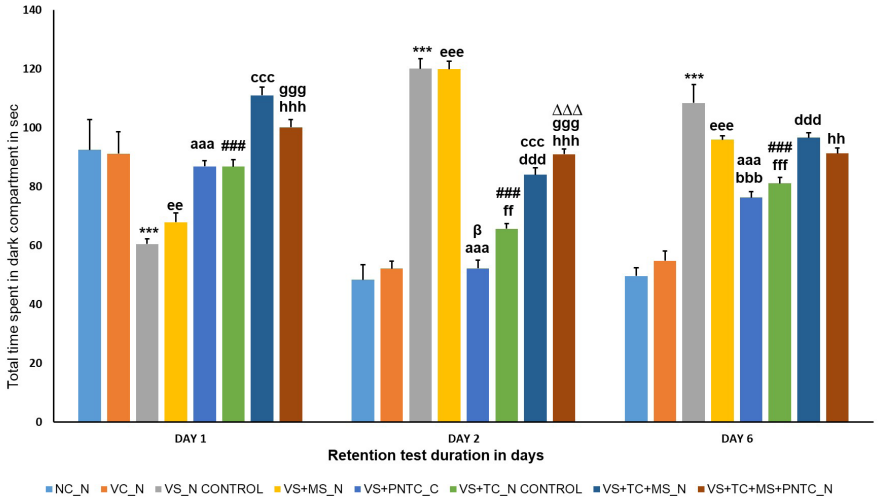

Figure 4. Total time neonates spent in the dark compartment (Mean±SEM) in the passive avoidance test. NC_N: normal control neonates; VC_N: vehicle control neonates; VS_N control: prenatally vibratory stressed control neonates; VS+MS_N: prenatally vibratory stressed neonates subjected to maternal separation; VS+PNTC_N: prenatally vibratory stressed neonates subjected to postnatal *Tinospora cordifolia* (TC) treatment; VS+TC_N: prenatally vibratory stressed and TC-treated neonate controls; VS+TC+MS_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation; VS+TC+MS+PNTC_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and postnatal TC treatment. *** $P < 0.001$ NC_N vs VS_N; ### $P < 0.001$VS_N control vs VS+TC_N control; β $P < 0.05$ VS+TC_N control vs VS+PNTC_N; aaa: $P < 0.001$ VS_N control vs VS+PNTC_N; bb $P < 0.01$, bbb $P < 0.001$ NC_N vs VS+PNTC_N; ccc $P < 0.001$ VS+MS_N vs VS+TC+MS_N; ddd $P < 0.001$ NC_N vs VS+TC+MS_N; ee $P < 0.01$, eee $P < 0.001$ NC_N vs VS+MS_N; ff $P < 0.01$, fff $P < 0.001$ NC_N vs VS+TC_N control; ggg $P < 0.001$ VS+MS_N vs VS+TC+MS+PNTC_N; hh: $P < 0.01$, hhh: $P < 0.001$ VS_N control vs VS+TC+MS+PNTC_N (Bonferroni post hoc correction).

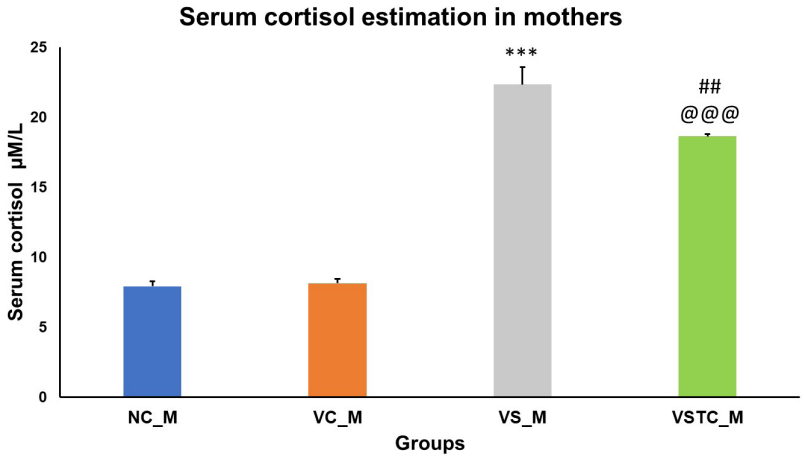

Figure 5. Results of serum cortisol estimation in mothers (Mean±SEM). NC_M: normal control mothers; VC_M: vehicle control mothers; VS_M: vibratory stressed mothers; VS+TC_M: vibratory stressed mothers with *Tinospora cordifolia* (TC) treatment. ***$P < 0.001$ NC_M vs VS_M; ## $P < 0.01$ VS_M vs VS+TC_M; @@@ $P < 0.001$ NC_M vs VS+TC_M (Bonferroni post hoc correction).

cortisol in the VS_N control and VS+MS_N neonates than in the control group (Figure 6). Compared with those of stressed neonates, the neonates in the VS+TC_N control and VS+PNTC_N groups showed decreased serum cortisol levels both during prenatal and postnatal TC treatment, indicating the antistress effects of TC treatment.

Dendritic morphology of amygdaloid neurons

The results of the quantification of amygdaloid neuronal dendritic arborization in mothers

Amygdaloid neuronal dendritic branching points were significantly ($P < 0.001$) greater in TC-treated mothers than in stressed mothers, with a greater number of branching points in the inner zones (20-60 µm) (Figure 7).

Compared with the animals in the VS_M group, the animals in the VS+TC_M group exhibited greater numbers of dendritic intersections at 20-40 µm intervals (Figure 8). Compared with control and stressed mothers, the TC-treated mothers showed increased dendritic branching points and intersections even in the outer zones (80-120 µm).

The consistent increase in the dendritic branching points and intersections throughout the length of neurons

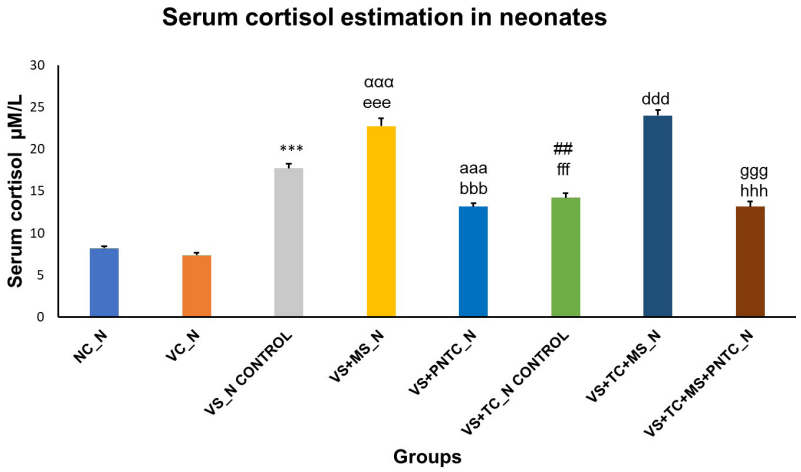

Figure 6. Results of serum cortisol levels in neonates (Mean±SEM). NC_N: normal control neonates; VC_N: vehicle control neonates; VS_N control: prenatally vibratory stressed control neonates; VS+MS_N: prenatally vibratory stressed neonates subjected to maternal separation; VS+PNTC_N: prenatally vibratory stressed neonates subjected to postnatal *Tinospora cordifolia* (TC) treatment; VS+TC_N: prenatally vibratory stressed and TC-treated neonate controls; VS+TC+MS_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation; VS+TC+MS+PNTC_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and postnatal TC treatment. *** $P<0.001$ NC_N vs VS_N; ## $P<0.01$ VS_N control vs VS+TC_N control; ααα $P<0.001$ VS_N control vs VS+MS_N; aaa $P<0.001$ VS_N control vs VS+PNTC_N; bbb $P<0.001$ NC_N vs VS+PNTC_N; ddd $P<0.001$ NC_N vs VS+TC+MS_N; fff $P<0.001$ NC_N vs VS+TC_N control; ggg $P<0.001$ VS+MS_N vs VS+TC+MS+PNTC_N; hhh $P<0.001$ VS_N control vs VS+TC+MS+PNTC_N. (Bonferroni post hoc correction).

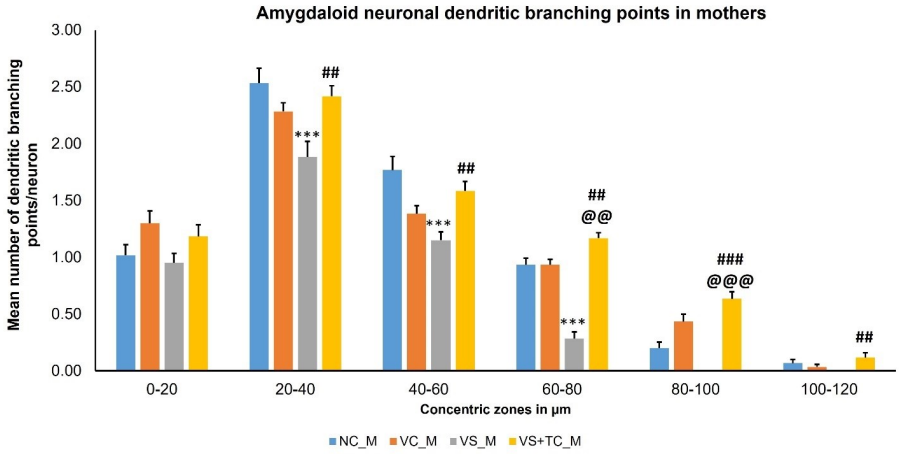

Figure 7. Amygdaloid neuron dendritic branching points (Mean±SEM) of mothers. NC_M: normal control mother; VC_M: vehicle control; VS_M: vibratory stressed mother; VS+TC_M: vibratory stressed along with *Tinospora cordifolia* (TC)-treated mother. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ NC_M vs VS_M; # $P<0.05$, ## $P<0.01$, and ### $P<0.001$ VS_M vs VS+TC_M; @@ $P<0.01$ and @@@ $P<0.001$ NC_M vs VS+TC_M. (Bonferroni post hoc correction).

in the TC-treated mothers indicated well-established synaptic connections in the amygdala (Figure 9).

The results of the quantification of amygdaloid neuronal dendritic arborization in neonates

Interestingly, compared with TC-treated neonates, the prenatally stressed (VS_N control) and double stressed (VS+MS_N) neonates exhibited significantly ($P<0.001$) increased dendritic branching points. However, these dendrites seldom intersected the 80 µm or 100 µm circles (Figure 10).

TC-treated neonates showed a significant ($P<0.01$) increase in dendritic branching points in the inner circles (20-60 µm). However, increased branching points were observed in postnatal TC-treated neonates (VS+PNTC_N) compared to the prenatal TC-treated neonates (VS+TC_N control). These groups also showed branching points even in the outermost zone (80-100 µm). A significant ($P<0.001$) increase in the dendritic intersections was observed in TC-treated neonates in the 20-60 µm circles compared to those in the VS_N control and VS+MS_N groups. Both prenatal TC-treated (VS+TC_N control) and postnatal TC-treated (VS+PNTC_N) neonates showed significantly greater intersections at the outermost circle

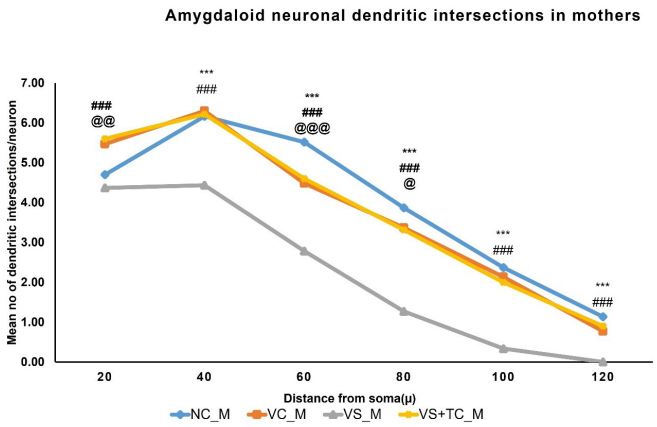

Figure 8. Amygdaloid neuron dendritic intersections (Mean±SEM) of mothers. NC_M: normal control mother; VC_M: vehicle control; VS_M: vibratory stressed mother; VS+TC_M: vibratory stressed along with *Tinospora cordifolia* (TC)-treated mother. *** $P < 0.001$ NC_M vs VS_M; ### $P < 0.001$ VS_M vs VS+TC_M; @ $P < 0.05$, @@ $P < 0.01$, and @@@ $P < 0.001$ NC_M vs VS+TC_M (Bonferroni post hoc correction).

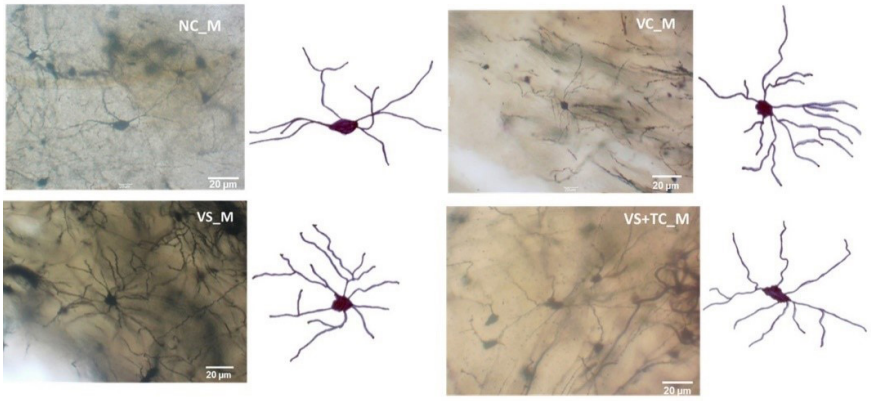

Figure 9. Representative photomicrographs of Golgi-stained amygdaloid neurons and their tracings using Sholl's method. A relative increase in dendritic arborization can be noted in the *Tinospora cordifolia* (TC)-treated groups compared to the stress-induced groups. NC_M: normal control mothers; VC_M: vehicle control mothers; VS_M: vibratory stressed mothers; VS+TC_M: vibratory stressed mothers and TC-treated mothers.

(120 μm) than the stressed neonates (Figure 11).

Increased amygdaloid dendritic branching points in the VS_N control and VS+MS_N groups indicated stress-induced amygdaloid neuronal hypertrophy but these dendrites were pruned and restricted to the innermost zone (Figure 12).

Analysis of the correlation between serum cortisol levels and memory retention power

Correlation between serum cortisol levels and latency to enter the dark compartment during the passive avoidance test

Compared to those in control and VS+TC_M group animals, the increase in serum cortisol levels in the VS_M group was directly correlated with a shorter latency to enter the dark compartment, suggesting that the increase in glucocorticoids in these animals could impair their ability to retain the memory of aversive stimuli received in the dark compartment.

Correlation between the serum cortisol level and latency to enter the dark compartment during the passive avoidance test in neonates

There was a strong linear correlation ($P < 0.01$) between the increase in the serum cortisol concentration and the latency to enter the dark compartment between the VS_N control and NC_N groups. In comparison to prenatally stressed (VS_N control) and double stressed (VS+MS N) neonates, prenatally stressed and TC-treated neonates (VS+TC_N control) showed a decrease in the serum cortisol level and a longer latency to enter the dark compartment. The VS+MS_N (double stressed) group displayed higher serum cortisol levels and a longer latency period than the VS_N control group. There was a linear correlation between the VS+TC_N control group and both postnatally TC-treated groups (VS+PNTC_N and VS+TC+MS+PNTC_N). The fact that neonates treated with TC had lower serum cortisol levels and longer latency times than those in the VS_N control

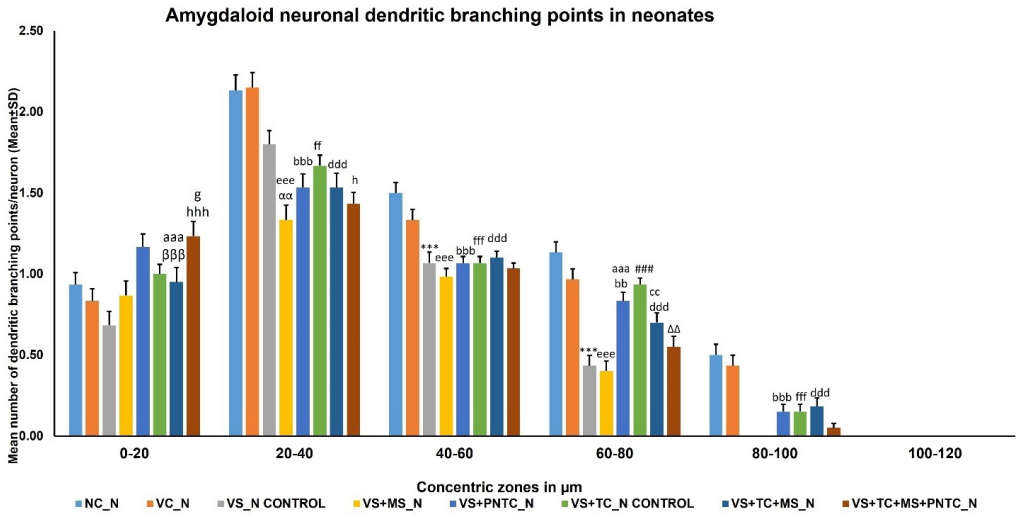

Figure 10. Amygdaloid neuronal dendritic branching points of neonates (Mean ± SEM). NC_N: normal control neonates; VC_N: vehicle control neonates; VS_N control: prenatally vibratory stressed control neonates; VS+MS_N: prenatally vibratory stressed neonates subjected to maternal separation; VS+PNTC_N: prenatally vibratory stressed neonates subjected to postnatal *Tinospora cordifolia* (TC) treatment; VS+TC_N prenatally vibratory stressed and TC-treated neonate controls; VS+TC+MS_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation; VS+TC+MS+PNTC_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and postnatal TC treatment. *** $P < 0.001$ NC_N vs VS_N control; ### $P < 0.001$ VS_N control vs VS+TC_N control; aaa $P < 0.001$ VS_N control vs VS+MS_N; bb $P < 0.01$ and bbb $P < 0.001$ NC_N control vs VS+PNTC_N; cc $P < 0.01$ VS+MS_N vs VS+TC+MS_N; ddd $P < 0.001$ NC_N vs VS+TC+MS_N; eee $P < 0.001$ NC_N vs VS+MS_N; ff $P < 0.01$ and fff $P < 0.001$ NC_N vs VS+TC_N control; g $P < 0.05$ VS+MS_N vs VS+TC+MS+PNTC_N; h $P < 0.05$, hhh $P < 0.001$ VS+_N Control vs VS+TC+MS+PNTC_N; αα $P < 0.01$ VS_N control vs VS+MS_N; βββ $P < 0.001$ VS+TC_N control vs VS+PNTC_N; ΔΔ $P < 0.01$ VS+PNTC_N vs VS+TC+MS+PNTC_N. (Bonferroni post hoc correction).

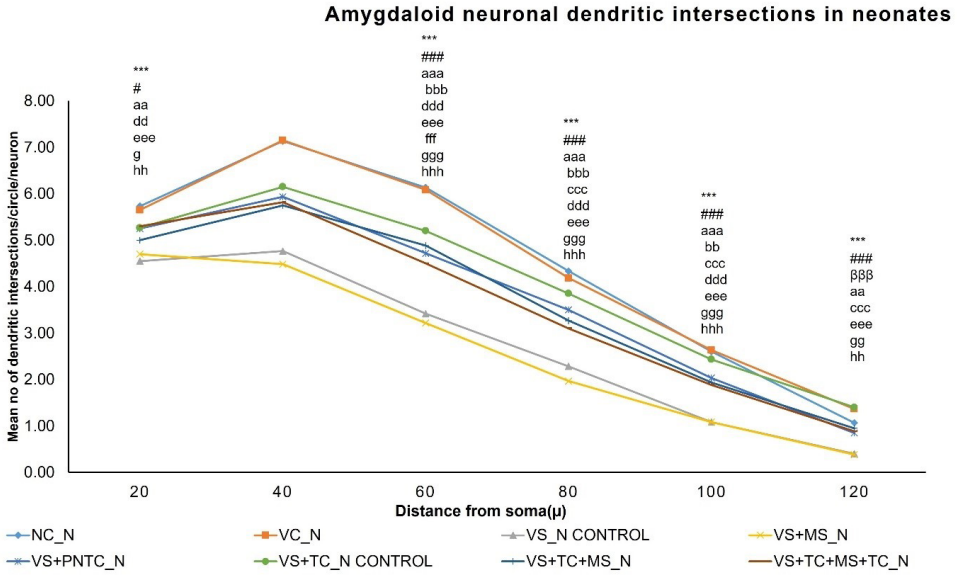

Figure 11. Amygdaloid neuronal dendritic intersections of neonates (Mean ± SEM). NC_N: normal control neonates; VC_N: vehicle control neonates; VS_N control: prenatally vibratory stressed control neonates; VS+MS_N: prenatally vibratory stressed neonates subjected to maternal separation; VS+PNTC_N: prenatally vibratory stressed neonates subjected to postnatal TC treatment; VS+TC_N: prenatally vibratory stressed and *Tinospora cordifolia* (TC)-treated neonate controls; VS+TC+MS_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and VS+TC+MS+PNTC_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and postnatal TC treatment. *** $P < 0.001$ NC_N vs VS_N control; # $P < 0.05$ and ### $P < 0.001$ VS_N control vs VS+TC_N control; aa $P < 0.01$ and aaa $P < 0.001$ VS_N control vs VS+MS_N; bb $P < 0.01$ and bbb $P < 0.001$ NC_N control vs VS+PNTC_N; ccc $P < 0.001$ VS+MS_N vs VS+TC+MS_N; dd $P < 0.01$ and ddd $P < 0.001$ NC_N vs VS+TC+MS_N; eee $P < 0.001$ NC_N vs VS+MS_N; fff $P < 0.001$ NC_N vs VS+TC_N control; g $P < 0.05$, gg $P < 0.01$, and ggg $P < 0.001$ VS+MS_N vs VS+TC+MS+PNTC_N; hh $P < 0.01$ and hhh p<0.001 VS+_N control vs VS+TC+MS+PNTC_N; βββ $P < 0.001$ VS+TC_N control vs VS+PNTC_N (Bonferroni post hoc correction).

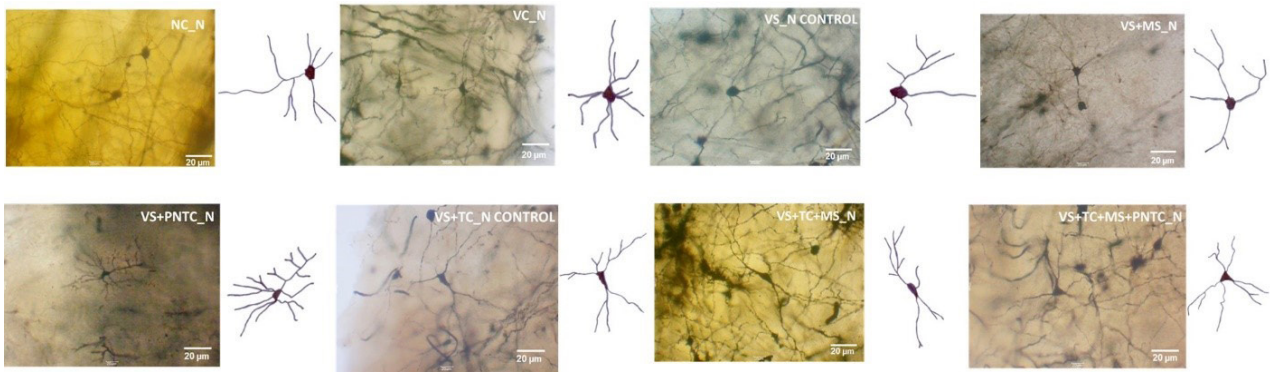

Figure 12. Representative photomicrographs of Golgi-stained amygdaloid neurons and their tracings using Sholl's method. A relative increase in dendritic arborization can be noted in *Tinospora cordifolia* (TC)-treated groups compared that of the stress-induced groups. NC_N: normal control neonates; VC_N: vehicle control neonates; VS_N Control: prenatally vibratory stressed control neonates; VS+MS_N: prenatally vibratory stressed neonates subjected to maternal separation; VS+PNTC_N: prenatally vibratory stressed neonates subjected to postnatal TC treatment; VS+TC_N control: prenatally vibratory stressed and TC-treated control neonates; VS+TC+MS_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation; VS+TC+MS+PNTC_N: prenatally vibratory stressed and TC-treated neonates subjected to maternal separation and postnatal TC treatment.

and VS+MS_N groups may suggest the anti-stress and memory-improving properties of TC.

Discussion

Brain development is affected by prenatal and early-life stress. Such stress may result in changes in amygdaloid neuronal connectivity and neuropsychiatric disorders in offspring (22,23). Earlier researchers have used various psychological stress models, such as maternal separation, exposure to predators, noise, and circadian variations. Physical stress models include foot shock, immobilization, exposure to temperature variation, and unpredictable stress (24,25). We established a new stress model to imitate the vibrations experienced by pregnant women during their day-to-day activities regularly, which influences the brain development of their offspring. In our study, we observed the effect of prenatal vibratory stress and maternal separation on the neuronal architecture of the amygdala and the memory retention and retrieval capacity of neonates exposed to these stressors.

The passive avoidance behavioral task assesses the retention of learned behavior (26). In the passive avoidance task, prenatally stressed (VS_N control) and double stressed (VS+MS_N) neonates showed longer latencies and spent more time in the bright compartment than the control and TC-treated neonates. This altered anxious behavior of neonate contrasts with their preference for darkness, suggesting alterations in the encoding and memory formation circuit due to prenatal vibratory stress and maternal separation. The longer latency and shorter time spent in the dark compartment by the TC-treated mothers than by the stressed mothers during the retention test after 24 hours and on the 5th day of foot shock suggested the retrieval and retention of memory. Prenatal TC-treated (VS+TC_N control) and postnatal TC-treated (VS+PNTC_N) neonates had longer latency times and spent less time in the dark compartment in the retention test after 24 hours and on the 5th day of foot shock than the stressed (VS_N control and VS+MS_N) and control groups. There are reports of TC-suppressing behavior similar to anxiety and enhancing cognitive function in rats deprived of sleep (27). Aqueous and alcoholic extracts of TC are known to improve memory retention in cyclosporine-induced memory deficits (28). Daily treatment with TC enhanced verbal learning, memory, and logical memory in healthy volunteers (29).

Earlier findings in our study revealed improved hippocampal spatial learning in these TC-treated neonatal groups. This implies that TC helped to mitigate the adverse effect of prenatal vibratory stress and maternal separation by establishing improved connectivity between the hippocampus and amygdala, the major centers for learning, memory formation, and recall.

The amygdala plays a vital role in the regulation of emotional behavior, including depression, fear, and anxiety (30-32). Recent investigations have revealed that acute and chronic maternal stresses alter spine density, dendritic length, and branching in the amygdala, hippocampus, prefrontal cortex, and other subcortical regions of the offspring brain (33). The developing amygdala is known to be highly susceptible to antenatal maternal cortisol, an endocrine indicator of stress (2,34). Many studies support the association between reduced neonatal amygdala volume and maternal depressive behavior (35,36). Maternal deprivation accelerates amygdala-prefrontal cortex functional development by increasing glucocorticoids, which indicates a strong relationship between the HPA axis and the amygdala (37). Studies have also suggested that the amygdala strongly influences the hippocampus during chronic stress, which indicates the possibility of rewiring the amygdala-hippocampal network and altering spatial learning and memory circuits (38).

In our study, a significant increase in the serum cortisol

level in prenatally stressed mothers (VS_Ms) suggested an altered HPA axis. Prenatally stressed and TC-treated mothers (VS+TC_Ms) who showed increased dendritic arborization in inner zones (20-80 μm) as well as outer zones (80-120 μm), compared to that of VS_Ms, revealed the potential of TCs to act against maternal cortisol-induced amygdaloid plasticity.

Relatively high levels of serum cortisol were observed in the VS_N control, VS+MS_N (double stressed), and VS+TC+MS_N neonatal groups, which might suggest the inhibition of glucocorticoid-metabolizing enzymes and the diffusion of maternal glucocorticoids through the placenta (7). TC significantly decreased the serum cortisol concentration in the VS+TC_N control and VS+PNTC_N groups.

The increased dendritic branching points in VS_N control and VS+MS_N (double stressed) neonates, observed in the inner zones (20-60 μ) caused by amygdaloid neuronal hypertrophy, suggested that prenatal vibratory stress and maternal separation accelerated neuronal growth. However, these neurons seldom intersect the outer circles (40-80 μm), indicating altered neuronal activity in the amygdala circuitry. Earlier studies suggested that the butanol extract of TC had neuroprotective effects by preventing neuronal degeneration caused by glutamate excitotoxicity by increasing dendritic growth in the limbic lobe; pretreatment with this extract inhibited glutamate-induced inflammation, stress, and mitochondrial damage (39). The significant increase in dendritic branching points and intersections in the outer zones in the VS+TC_N control, VS+PNTC_N, VS+TC+MS_N, and VS+TC+MS+PNTC_N groups could suggest the neuroprotective potential of TC.

There are studies suggesting that medicinal drugs such as *Centella asiatica* (20), *Bacopa monnieri* (40), *Withania somnifera*, *Clitoria ternatea*, *Emblica officinalis, and Ocimum sanctum* (41,42) have the potential to improve spatial learning and memory. Treatment with fresh leaf extract of *Centella asiatica* reportedly improves memory formation and memory retrieval and improves amygdaloid neuronal architecture during the growth spurt period in rats (43,44). *Bacopa monnieri* has been proven to promote hippocampal neurogenesis (45), enhance the dendritic arborization of amygdaloid neurons (46), and improve cognitive functions (40,47,48). With these considerations in mind, we investigated the correlation between serum cortisol levels and latency to enter the dark compartment during avoidance learning behavior in stressed and TC-treated animals.

The strong linear correlation observed between the increased serum cortisol concentration and decreased latency to enter the dark compartment in the stressed mothers (VS_M) compared to those in the control and VS+TC_M groups suggested that vibratory stress may affect the formation and retention of memories in these animals. There was a significant ($P<0.01$) correlation between cortisol level and latency time between control (NC_N) and prenatally stressed neonates (VS_N control). An increase in serum cortisol also significantly ($P<0.05$) increased the latency time to enter the dark compartment in the VS_N control and VS+MS_N (double stressed) groups. However, a positive linear correlation was observed between prenatal and postnatal TC-treated neonatal groups, suggesting that TC might help to reduce serum cortisol levels and improve retention memory in prenatally stressed and postnatal maternal separation neonatal group rats.

The primary objective of the present study was to explore the neuroprotective potential of TC extract on prenatal vibratory stress and maternal separation-induced neuronal plasticity. However, it is crucial to address several limitations inherent in our research methodology that may impact the interpretation of our findings.

- The vibratory stress model utilized in our study is relatively novel; there is a scarcity of references guiding the precise methods for inducing such stress in the literature. The process of standardizing the stress induction protocols required considerable time and effort due to the limited existing literature guidance.
- The implementation of vibratory stress on pregnant rats from specific gestational days posed challenges related to in-house breeding and accurately determining the exact gestational periods. These logistical hurdles may have introduced variability in stress exposure across subjects, potentially influencing the study outcomes.
- While numerous research studies have highlighted the neuroprotective properties of TC extract in preclinical models, there is a noticeable gap in the literature regarding its efficacy in human subjects. Although there is a growing body of evidence supporting its benefits, particularly in animal models, the extrapolation of these findings to human populations remains a topic of ongoing investigation and discussion.

Despite these limitations, we have endeavored to rigorously design and execute our study, implementing quality control measures wherever possible to mitigate biases and ensure the reliability of our results. By acknowledging these limitations transparently, we aim to encourage a critical evaluation of our findings and contribute to the ongoing discourse on potential therapeutic applications of TC extract in neuroprotection.

Conclusion

Stressful experience during the gestation period is known to increase maternal cortisol levels, which affects amygdaloid neuronal proliferation and memory formation in neonates. Prenatal stress and maternal isolation during a critical period for learning have a significant impact on fetal brain cognitive development. The improved

dendritic arborization of amygdaloid neurons in prenatal and postnatal TC-treated neonates suggested that TC had neuroprotective effects on prenatal vibratory stress and maternal separation. The improved performance of TC-treated animals in the passive avoidance task suggested that both mothers and neonates improved memory retention power.

The results of the present study suggest that the TC might be used early in pregnancy to treat maternal depression and anxiety disorders to improve fetal brain development. Therapeutic application of TC extracts in early infancy might enhance neurogenesis and ameliorate the effect of maternal separation in children born to working-class women.

Authors' contributions

Conceptualization: Mohandas Rao KG, Ashwini LS.
Data curation: Ashwini LS.
Formal analysis: Ashwini LS.
Investigation: Ashwini LS.
Methodology: Mohandas Rao KG, Ashwini LS, Guruprasad Rao, Padmanabha Udupa, Somashekar B Shetty, Chinmay Ajit Suryavanshi.
Project administration: Mohandas Rao KG, Kiranmai S Rai.
Resources: Guruprasad Rao, Padmanabha Udupa, Somashekar B Shetty, Chinmay Ajit Suryavanshi
Software: Ashwini LS, Chinmay Ajit Suryavanshi.
Supervision: Mohandas Rao KG, Kiranmai S Rai, Somashekar B Shetty.
Validation: Mohandas Rao KG, Kiranmai S Rai, Somashekar B Shetty, Guruprasad Rao, Padmanabha Udupa.
Visualization: Mohandas Rao KG, Ashwini LS.
Writing–original draft: Ashwini LS.
Writing–review & editing: Mohandas Rao KG, Ashwini LS.

Conflict of interests

None.

Ethical considerations

The protocol was approved by Institutional Animal Ethical Committee, Manipal Academy of Higher Education, Manipal, India (Approval letter Reference No.: IAEC/KMC/56/2015).

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