Neuroprotective Efficacy of *Clitoria Ternacea* Root Extract on Hippocampal CA3 Neurons – A Quantitative Study in Mice

Shetty Hemamalini[1] Muddanna S. Rao[2]

[1] Department of Anatomy, JSS Medical College, Mysore, Karnataka, India
[2] Department of Anatomy, Faculty of Medicine, Kuwait University, Safat, Kuwait

Address for correspondence Dr. Hemamalini, MD, Assistant Professor, Department of Anatomy, JSS Medical College, Mysore – 570015, Karnataka, India (e-mail: shettyhemamalini@gmail.com).

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Abstract

Clitoria ternatea is a vigorous, herbaceous perennial legume that belongs to the Fabaceae family. All parts of the plant are used in the preparations of Ayurvedic drugs. It is an astringent, an aphrodisiac, a rejuvenator, and a brain tonic. It also has anti-inflammatory, analgesic, and antipyretic properties. Baidyanath Shankapushpi, which contains extracts of herbs such as *C. ternatea*, *Bacopa monnieri*, *Withania somnifera* and *Asparagus racemosus*, is clinically administered for memory improvement, blood purification and to improve digestion. However, its neuroprotective effect has not been reported so far. In the present study, the neuroprotective effect of *C. ternatea* root (CTR) extract on hippocampal CA3 neurons was investigated. Three-month-old albino mice were divided into four groups. Group I was the normal control, group II was the saline control, group III was the stress group, and group IV was the stress + CTR-treated group. Group-III mice were stressed in a wire mesh restrainer for 6 hours/day for 6 weeks. Grou-IV mice were also stressed like group III, but received CTR extract orally throughout the stress period. After 6 weeks, their brain was removed, and their hippocampi were dissected and processed for Golgi staining. The hippocampal neurons were traced using a camera lucida focused at 400x magnification. The Sholl concentric circle method was used to quantify the dendrites. The results showed a decrease in the number of dendritic branching points and of dendritic intersections in the stressed group. On the other hand, there was an increase in the number of dendritic branching points and of dendritic intersections of hippocampal CA3 neurons in group IV, which was subjected to restraint stress and was treated with the CTR extract. The results showed that the oral administration of CTR significantly increased the dendritic branching points and the dendritic intersections of hippocampal CA3 neurons.

Keywords
- restraint stress
- dendritic branching points
- dendritic intersections
- hippocampus

Introduction

The hippocampus belongs to the limbic system and plays important roles in the integration of information from the short-term memory to the long-term memory and to the spatial memory. The hippocampus is one of the important areas of the brain concerned with learning, memory, and emotional behavior of the individual.[1] It is also involved in the control of adrenocorticotropic hormone (ACTH) secretion through the hypothalamo-pituitary-adrenal (HPA) axis.[2]

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Damage to the neurons of the hippocampus is seen in some neurological disorders, such as Alzheimer disease and epilepsy.[3,4] People with extensive, bilateral hippocampal damage may experience amnesia – the inability to form and retain new memories.

Neurons are the excitable cells of the nervous system. They are highly specialized for the processing and transmission of nerve signals. Neurons communicate by chemical and electrical synapses via neurotransmission, with the release of neurotransmitters. The structure, the neurochemical composition, and the physiological activity of the neurons can be altered by several factors. The neuronal structural organization, particularly the dendritic arborization and the synaptic junctions, respond to several factors, such as stress, ultrasound and pesticides.[5–7]

On the other hand, various experimental studies on mice and rats have shown that the administration of extracts of certain plants, such as *Centella asiatica, C. ternatea,* and *Ocimum* sanctum,[8–10] results in an improvement in the dendritic arborization.

Chronic exposure to stress results in hippocampal neuronal death.[11] The effect of restraint stress on the hippocampal CA3 neurons has been reported as leading to dendritic atrophy.[12] The extract of fresh *C. ternatea* roots (CTRs) at a dose of 100 mg/kg/day for 30 days showed a significant improvement in the learning ability of rats.[13] This was correlated with increased dendritic branching points and dendritic intersections in hippocampal CA3 neurons.[14] But the anti-stress effect of *C. ternatea* on the dendritic branching points and on the intersections of the hippocampal CA3 neurons has not been reported. Our aim was to determine the anti-stress effect of the CTR extract on the dendritic branching points and on the intersections of hippocampal CA3 neurons.

Materials and Methods

Three-month old male and female albino mice weighing between 30 g and 36 g were included in the present study. The mice were bred and maintained in the central animal house. The mice were maintained in 12-hour light and 12-hour dark cycles in a well-ventilated room. Four to six mice were housed in each polypropylene cage. Paddy husk was used as the bedding material, which was changed on alternate days. The mice were given ad libitum access to food and water, except during the stress period of the experimental study.

Extraction Procedure

Fresh CRTs were collected, cleaned, sunshade-dried, and then powered. The dry powder was weighed and mixed with distilled water at a 1:10 ratio, and boiled over a low flame for 30 minutes. The solution was cooled and decanted. This procedure was repeated twice. The clear supernatant obtained each time was decanted and then centrifuged (300 rpm for 5 minutes). The supernatant was evaporated on a low flame to get a thick paste-like extract, which was later dried in a desiccator.

Drug Dosage

The dry CTR extract was prepared and stored in an air tight bottle. For each mouse, 100 mg/kg body weight of CTR extract was administered orally in separate groups throughout the experimental period (6 weeks). The dose was standardized from previous publications from our laboratory and preliminary studies using different doses. The CTR extract was dissolved in a saline solution to get the appropriate dilution to be administered orally just before the stress exposure on each day.

Oral Intubation

The required dose of the drug was put in a syringe attached to a capillary tube, and the tube was introduced gently into the oral cavity of the mice to ensure a slow delivery of the drug.

Restrainer and Stress Procedure

A locally-fabricated wire mesh restrainer consisting of 12 compartments was used for restraint stress (►**Fig. 1**). Each compartment has a dimension of 2" (length) X 1.5" (breadth) X 1.4" (height). The mice were stressed individually by being placed inside the restrainer for 6 hours/day for 6 weeks. The stress induction and its severity were assessed by measuring the suprarenal gland weight at the time of sacrifice.

Experimental Design

The mice were divided as follows:

Group I: normal control group (NC) - remained undisturbed in their home cage.
Group II: saline control group (SC) – received an equivolume of normal saline solution during the experimental period (6 weeks).
Group III: stress group (S) – stressed in a wire mesh restrainer 6 hours/day for 6 weeks.
Group IV: stress + *C. ternatea* group (S + CTR) – stressed in the same way as group III, and treated with 100 mg/kg/day of aqueous CTR extract throughout the stress period (6 weeks). The drug was administered orally just before stress exposure on each day.

A day after the last dose or on the equivalent day in the control group, all the mice in all the groups were sacrificed with ether anesthesia. Their brains were removed, and their hippocampus were dissected and processed for rapid Golgi staining ($n = 8$ in each group). The number of dendritic branching points and of dendritic intersections were quantified (►**Fig. 2**).

Selection of Neurons for Quantification

The slides were viewed through a compound microscope attached with a camera lucida apparatus. The tracing of the neurons was made using the camera lucida focused at 400x magnification. The Sholl concentric circle method was used to quantify the dendrites. The concentric circles were drawn at 20 µm intervals on a transparent sheet and used for dendritic analysis. The center of the cell body was taken as the reference point. Using the camera lucida tracings of the neurons, the following analyses were performed:

Fig. 1 Wire mesh restrainer with mice. The mice were stressed in this restrainer for 6 hours/day for 6 weeks.

Dendritic Branching Points

This is a measure of the nature of the dendritic arborization. The number of dendritic branching points within each concentric circle (between adjacent concentric circles) was counted.

Dendritic Intersections

This is a measure of the total length of the dendrites. The dendritic intersection is a point in which a dendrite touches or crosses a concentric circle of the Sholl grid placed over a traced neuron.

Statistical Data Analysis

Data obtained from the aforementioned experiments were correlated and analyzed using one-way analysis of variance (ANOVA), followed by the Bonferroni posttest. The student *t*-test was applied whenever suitable using a statistical software package (InStat, GraphPad Software, San Diego, CA, US).

Results

Dendritic Branching Points

Apical Dendritic Branching Points at Different Concentric Zones

The apical dendritic branching points decreased in the stressed group in all concentric zones compared with the NC group. In the S + CTR group, the dendritic branching

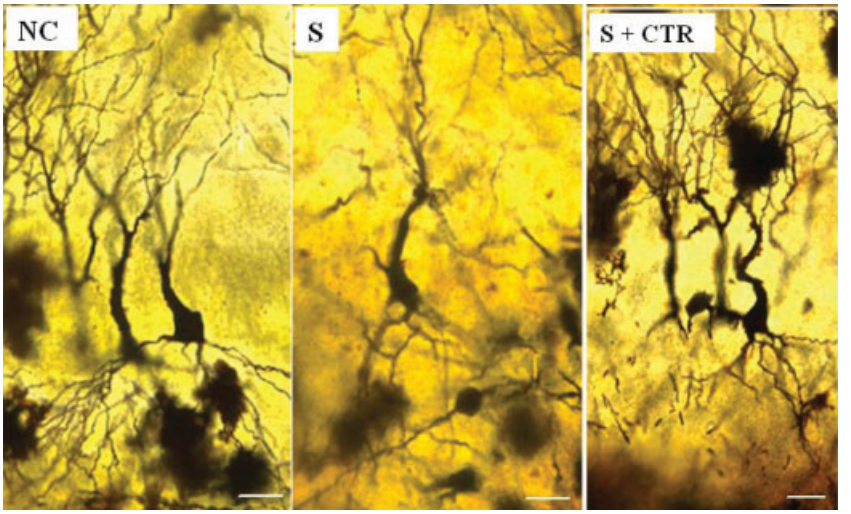

Fig. 2 Photomicrographs of Hippocamal CA3 Neurons (Golgi staining) in different groups. NC – Normal control, S – Stressed, S + CeA – Stressed and treated with *Centella asiatica* leaf extract. Scale bar = 40 µm. Note the increased dendritic arborization of the hippocampal CA3 neurons in the S + CeA – treated group compared with the stressed group.

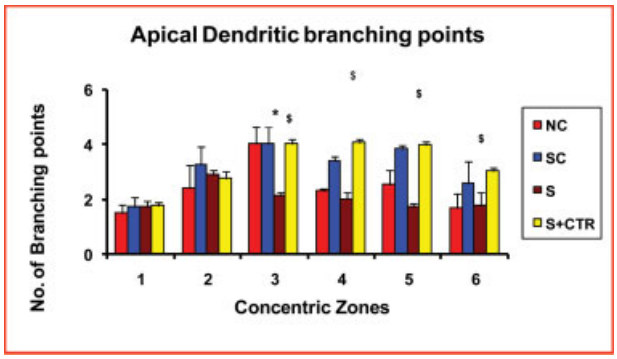

Fig. 3 Effect of the *Clitorea tematea* root extract on the apical dendritic branching points of CA3 neurons.

points between the 40 µm and 60 µm, 60 µm and 80 µm, 80 µm and100 µm, and 100 µm and 120 µm concentric zones were significantly increased compared with the S group ($p < 0.05$–0.001; one-way ANOVA; Bonferroni test) (►**Fig. 3**).

Basal Dendritic Branching Points at Different Concentric Zones

The basal dendritic branching points decreased significantly in the S group between the 0 µm and 20 µm, 20 µm and 40 µm, 40 µm and 60 µm, and 60 µm and 80µm concentric zones compared with the NC group. In the S + CTR group, the dendritic branching points in all concentric zones were significantly increased compared with the S group ($p < 0.01$–0.001; one-way ANOVA; Bonferroni test) (►**Fig. 4**).

Dendritic Intersections

Apical Dendritic Intersections

The apical dendritic intersections decreased at the 60 µm, 80 µm, 100 µm, and 120 µm distances from the soma in the S and in the S + CTR groups compared with the NC and the SC groups ($p < 0.001$). The dendritic intersections were increased significantly in the same radial distances in the S + CTR group ($p < 0.001$) (►**Fig. 5**).

Basal Dendritic Intersections

The basal dendritic intersections decreased at the 20 µm, 40 µm, 60 µm and 80 µm distances from the soma in the S and

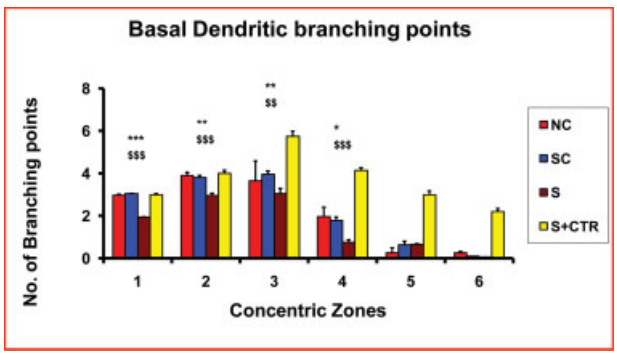

Fig. 4 Effect of the *Clitorea tematea* root extract on the basal dendritic branching points of CA3 neurons.

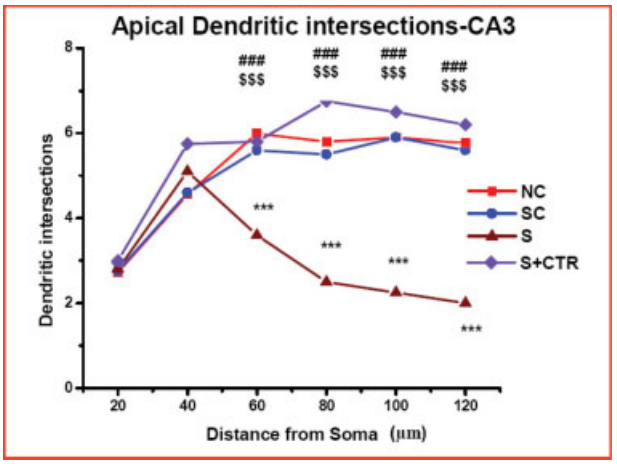

Fig. 5 Effect of the *Clitorea tematea* root extract on the apical dendritic intersections of hippocampal CA3 neurons.
Note: In the stressed group (S), there was a decrease in the apical dendritic intersections, which increased in the *Clitoria ternatea* root (CTR) extract-treated groups. Normal control (NC) vs. S - *** $p < 0.001$; S vs. CTR - $$$ $p < 0.001$ (one-way analysis of variance [ANOVA]; Bonferroni Test).

in the S + CTR groups compared with the NC and the SC groups ($p < 0.05$–0.01). The dendritic intersections increased significantly in the same radial distances in the S + CTR group ($p < 0.001$) (►**Fig. 6**).

Discussion

In the present study, there was atrophy in the dendritic arborization of the hippocampal CA3 neurons, which may affect several functions of the hippocampus. The possible mechanisms for this dendritic atrophy can be due to:

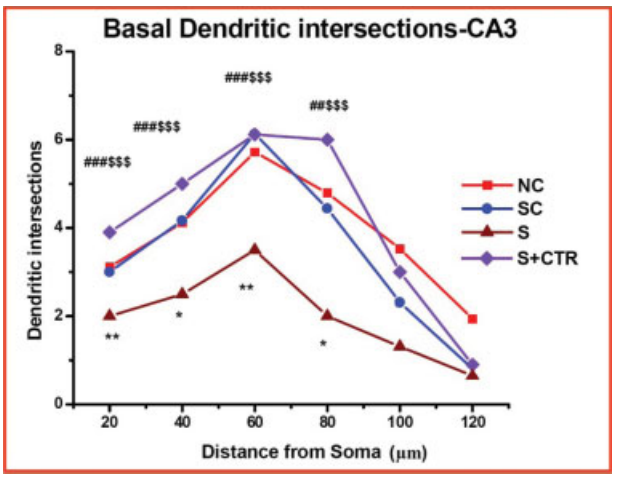

Fig. 6 Effect of the *Clitorea tematea* root extract on the basal dendritic intersections of hippocampal CA3 neurons.
Note: In the stressed group (S), there was a decrease in the basal dendritic intersections, which increased in the groups treated with the *Clitoria ternatea* root (CTR) extract. Normal control (NC) vs. S -* $p < 0.05$, ** $p < 0.01$; S vs. CTR - $$$ $p < 0.001$ (one-way analysis of variance [ANOVA]; Bonferroni Test).

Excitotoxicity

Excitotoxicity is a pathological condition in which neurons are damaged by an excessive release of the excitatory neurotransmitter glutamate. Glutamate may be responsible for the dendritic atrophy of the hippocampal CA3 neurons. Elevated levels of circulating glucocorticoids (GCs) increase the glutamate levels in the hippocampus.[14] Restraint stress has been shown to increase glutamate release in the hippocampus.[15]

Glucocorticoid Toxicity

Stress increases the levels of GCs, which cause atrophy of the hippocampal neurons. The findings of Dallman et al[16] reveal that, during stress, the enhanced activity of the adrenocortical axis elevates the circulating concentrations of GCs. The hippocampus is a predominant neural target for GCs.[17]

Both stress and GCs increase glutamate concentrations in the hippocampal synapses.[18,19] Glucocorticoids selectively increase glutamate accumulation in response to excitotoxic insults both in hippocampal cultures and in the hippocampus in vivo.[12,20]

Glucocorticoids have long been known to inhibit glucose transport in various peripheral tissues. This can be viewed as a strategy to divert energy toward exercising the muscles during a stressor.[21] A similar inhibition has been reported in the hippocampus, with GCs decreasing the glucose uptake by cultured neurons and glia, and decreasing the local cerebral glucose utilization in the hippocampus in vivo.[22–24] Glucocorticoids accelerate the decline in the hippocampal concentrations, metabolism and mitochondrial potentials of adenosine 5'-triphosphate (ATP), during insults.[25]

Glucocorticoids enhance the calcium ion (Ca^{2+}) currents in the hippocampus.[25] It has been reported that GCs increase the cytosolic Ca^{2+} concentrations in cultured hippocampal neurons.[26,27] Glucocorticoids inhibit the transcription of the Ca^{2+}-ATPase pump, which plays a key role in extruding cytosolic Ca^{2+}.[28] Both GCs and stress worsen Ca^{2+}-dependent degenerative events, such as cytoskeletal proteolysis and tau immunoreactivity.[29]

Increased Levels of Calcium Ions

The influx of Ca^{2+}activates several enzymes. These enzymes damage cell components such as the cytoskeleton, the membrane and the DNA. The Ca^{2+}-activated neural protease (Calpain) is responsible for the breakdown of cytoskeletal proteins, disassembling the microtubules. This may lead to the collapse and the retraction of the dendritic branches, since the structural integrity of the neuronal processes depends on the presence of stable microtubules. Glucocorticoids increase the glutamate levels in the hippocampus, resulting in excitotoxicity, which, in turn, increases the cytosolic Ca^{2+} levels in the neurons.

Brain-derived Neurotrophic Factor

Corticosterone is known to regulate the intracellular localization of the brain-derived neurotrophic factor (BDNF) in the rat brain.[30] Stress and GCs have markedly reduced the BDNF levels in the dentate gyrus and in the hippocampus.[31] Corticosterone induces damage to cultured rat hippocampal neurons by reducing their BDNF synthesis, and this was attenuated by exogenously-added BDNF.[32]

Stress and GCs are reported to decrease the expression of BDNF in the hippocampus and in the dentate gyrus. Decreased levels of BDNF in response to stress could lead to the loss of normal plasticity and, eventually, to neuronal damage and loss.

The probable mechanisms involved in increasing the dendritic arborization of hippocampal CA3 neurons and in the protection against stress-induced neuronal injury are:

Neurostimulants and Synaptic Modulation

The CTR extract may contain neurostimulants, which may stimulate the formation of new dendrites, or may influence the hippocampus to release the corticotrophin-releasing factor, which, in turn, may increase the synaptic efficacy in the hippocampus. New synapse formation to compensate the neuronal loss due to stress has been reported.

Growth Factors, Adhesion Molecules and Other Chemicals

The injection of nerve growth factor (NGF) directly into the hippocampus improves spontaneous behavior and memory retention, which may occur by influencing the formation of new dendrites. The CTR extract may contain NGF-like substances.

Neurogenesis

The CTR extract may induce neurogenesis in the neural structures involved in stress, such as the hippocampus. Neurogenesis has been reported in the hippocampus in relation to learning or task training. Conversely, aversive experiences, such as stress, seem to decrease the production of new cells.

Neuroprotectors and Antioxidants

The aqueous CTR extract may act as an antioxidant and may have an enhancing effect on cognitive functions. The derivatives of Asiatic acid, a triterpene extracted from *C. Ternatea*, are efficacious in protecting neurons from oxidative damage caused by exposure to an excess of glutamate. Accordingly, in the present study, the cytoprotective and antioxidant properties of CTR may be responsible for the neuroprotection against cell death and the deleterious effects of stress.

Conclusion

The present study concludes that the oral intubation of the CTR extract in stressed albino mice leads to the following:

i. The CTR extract increased the apical and basal dendritic branching points of hippocampal CA3 neurons in stressed mice.
ii. The extract also increased the dendritic intersections of both apical and basal dendrites of hippocampal CA3 neurons.

Significance of the Study

The present study proves the neuroprotective effect of the CTR extract, which protects the neurons against stress-induced neuronal atrophy.

i. The CTR extract can be used to treat stress disorders.
ii. The CTR extract can also be used to prevent age-related, drug-induced, or spontaneous neurodegeneration.

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