Centella asiatica Leaves Extract Causes Permanent Changes in the Dendritic Architecture of Hippocampal CA3 Neurons in Albino Mice

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Received: 17-Jan-2024, Manuscript No. jnn-24-125161; **Editor assigned:** 20-Jan-2024, PreQC No: jnn-24-1125161(PQ); **Reviewed:** 31-Jan-2024, QC No. jnn-24-125161(Q); **Revised:** 12-Feb-2024, Manuscript No. jnn-24-125161(R); **Published:** 24-Feb-2024, DOI: 10.35248/2332-2594.24.15 (1).343

Abstract

Introduction: *Centella asiatica* (CeA) is an herbaceous perennial creeper, growing in moist soil belonging to the umbelliferae family. *Centella asiatica* is used as brain tonic in ayurvedic medicine to enhance the neural function, learning and memory. This was correlated with improved dendritic arborization in normal animals. But CeA leaves extract protecting the neurons from neurodegeneration induced by stress was not reported. So in the present study the neuroprotective effect of CeA leaves extract on hippocampal CA3 neurons in restraint stressed mice was investigated first and then the effect of rehabilitation in stress and stress + CeA extract treated mice.

Materials and Methods: Experiment I: Three months old albino mice were divided into four groups. Group (i) was normal control, Group (ii) was saline control, Group (iii) was stress group, Group (iv) was stress + CeA treated group. Group (iii) mice were stressed in a wire mesh restrainer for 6 hours/day for 6 weeks. Group (iv) mice were also stressed like group (iii) but they received orally CeA leaves extract throughout the stress period. After 6 weeks, brain was removed, hippocampus was dissected and processed for Golgi staining. Hippocampal neurons were traced using camera lucida focused at high magnification. The concentric circle method of Sholl was used to quantify the dendrites.

Experiment II - Rehabilitation experiment - done in the same manner as mentioned above followed by 30 days of rehabilitation in the normal laboratory conditions after the last dose of extract.

Result: There was significant decrease in the number of dendritic spines, dendritic branching points and dendritic intersections in the hippocampal CA3 neurons in group (iii) both in experiment I and experiment II even after 30 days after rehabilitation. However, there was significant increase in group (iv) which was subjected to restraint stress and treated with CeA leaves extract both in experiment I and after 30 days of rehabilitation (Experiment II).

Conclusion: CeA protected the hippocampus CA3 neurons against the stress induced neurodegeneration. CeA leaves extract caused permanent changes in the dendritic morphology of hippocampal CA3 neurons (Experiment II)..

Keywords: Restraint stress • Rehabilitation • Dendritic morphology • atrophy • Hippocampus

Introduction

Hippocampus is one of the important area of the brain concerned with learning and memory processing through long term potentiation which strengthens the synapses and long lasting increase in signal transmission between the neurons [1]. Hippocampus is also known for its remarkable neuronal plasticity and its involvement in some of the neurodegenerative diseases like dementia, Alzheimer's disease and epilepsy [2, 3].

Neurons are the structural and functional unit of the nervous system. The structural organization of the neurons, neurochemical composition and functional integrity is known to be altered by various factors. The structural organization particularly the dendritic morphology and synaptic junctions respond to various factors like stress, ultrasound and alcohol [4-6].

However, various experimental studies on rats have shown that there is an improvement in dendritic arborization by the intake of certain plant extracts like *Centella asiatica*, *Bacopa monniera* and *Glycyrrhiza glabra* [7-9].

Stress results in neuronal loss, structural and functional changes in hippocampus [10]. The effect of restraint stress on the hippocampal CA3 neurons has been reported to cause dendritic atrophy [11]. CeA leaves extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats [12]. Fresh leaves extract of CeA at a dose of 4 ml/kg/day and 6 ml/kg/day for 6 weeks has shown significant improvement in learning ability of rats. This was correlated with increased dendritic branching points and dendritic intersections in hippocampal CA3 neurons and amygdala neurons [13]. But the anti- stress effect of CeA on the dendritic branching points and intersections of the hippocampal CA3 neurons has not been reported. Thus, the aim of the study was to determine the anti-stress effect of CeA leaf extract on the neuron morphology (dendritic spine density, dendritic branching points and intersections) of hippocampal CA3 neurons and the effect of rehabilitation on stress and stress + CeA extract treated groups.

Methods

Experimental animals

The study procedure was approved by the Institutional Ethics Committee of KMC, Manipal University, India (No. IAEC/KMC/02/2002-2003, Dated Oct 21st 2002). Male and female albino mice of 3 months of age weighing 30 g-36 g were included in the present study. The mice were bred and maintained in the central animal house. The mice were maintained in light and dark cycles of 12 h duration each, in a well-ventilated room. Four to six mice were housed in each polypropylene cage, with paddy husk as the bedding material, changed on alternate days. Mice were fed with food and water ad libitum except during stress period of the experimental study.

Extraction procedure

Fresh *Centella asiatica* leaves were collected, cleaned, sunshade dried and powered. Dry powder was mixed with distilled water at 1:10 ratio and boiled over a low flame for 30 minutes. The solution was cooled and decanted. The procedure was repeated twice. 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

Dry CeA leaf extract was prepared and stored in air tight bottles. The CeA leaf extract of 500 mg/kg body weight was administered to mice, orally throughout the experimental period (6 weeks). The dose selected was the standardized dose from our preliminary study [12,13]. Plant extract was dissolved in distilled water to get the appropriate dilution to administer orally just before the stress exposure on each day.

Oral administration of drugs

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

Restrainer and stress procedure

A wire mesh restrainer, fabricated locally consisting of 12 compartments was used for restraint stress. Each compartment had 2" (length) × 1.5" (breadth) × 1.4" (height) dimension. Mice were stressed individually by placing within the wire mesh restrainer for 6 h/day for 6 weeks. Stress induction and its severity were assessed by measuring the suprarenal gland weight at the time of sacrifice. Chronic restraint stress, with wire mesh, for 6 h/21 days is a reliable and efficient method to produce psychological stress and to cause CA3 dendritic retraction in rats Figures 1A and 1B [14].

Experimental design

Experiment I: Effect of CeA leaves extract on hippocampal CA3 neurons in restraint stress mice.

Group (i) or the Normal Control (NC) remained undisturbed in their home cage. Group (ii) or the Saline Control group (SC) received equivolume of normal saline during the experimental period. Group (iii) was stressed (S) in a wire mesh restrainer 6 h/day for 6 weeks. Group (iv) was stressed in the same way as group (iii), but treated with CeA aqueous extract of 500 mg/kg/day throughout the stress period (S+CeA). Extract was administered orally just before the stress exposure on each day.

A day after the last dose or equivalent day in control group, mice in all the groups were sacrificed with ether anaesthesia. Brain was removed, hippocampus was dissected and processed for rapid Golgi staining (n=8 in each group). Hippocampal neurons were traced using camera lucida focused at high magnification. The concentric circle method of Sholl was used to quantify the dendrites. Number of dendritic branching points, dendritic intersections and dendritic spine density were quantified.

Experiment II: Rehabilitation experiment - done in the same manner as above followed by 30 days of rehabilitation in the normal laboratory conditions after the last dose of extract. A day after the rehabilitation period, mice in the all the groups were sacrificed with ether anaesthesia. Brain was removed and hippocampus was dissected and processed for Golgi staining. Hippocampal neurons were traced using camera lucida focused at high magnification. The concentric circle method of Sholl was used to quantify the dendrites. Number of dendritic branching points, dendritic intersections and dendritic spine density were quantified.

Quantification of neurons

Slides were viewed by using a compound microscope attached with the Camera lucida apparatus. Tracing of neurons were made using the Camera lucida focused at high magnification. The concentric circle method of Sholl was used to quantify the dendrites. Concentric circles were drawn at 20 µm intervals on a transparent sheet and used for dendritic analysis. The centre of the cell body was taken as the reference point. Using the camera lucida tracings of neurons following analysis were done.

Dendritic spine count

To quantify the dendritic spine, well stained hippocampal neurons were selected. The dendrites were classified as follows, the main apical dendrite arising from soma designated as main shaft, branch arising from main shaft as primary branch, branch arising from primary branch as secondary branch. In case of basal dendrites, the dendrite arising from the soma will be designated as primary dendrites and that from primary as secondary and from secondary as tertiary branch. In all types of dendrites, number of dendritic spines along a length of 100 µm was quantified using a calibrated ocular micrometer at high magnification. The spines were counted from the origin of the main shaft, primary and secondary in apical dendrites and primary, secondary and tertiary branches of basal dendrites as shown in the Figures 2A and 2B
In all dendritic quantification, from each animal 8-10 neurons were selected and group mean was calculated.

Statistical analysis

Results obtained from the above experiments were correlated and Analysed Using One Way Analysis of Variance (ANOVA) followed by Bonferroni's post-hoc test. Student's t-test was applied wherever applicable using statistical software package (Graph pad in Stat). A p-value of ≤ 0.05 was considered as statistically significant.

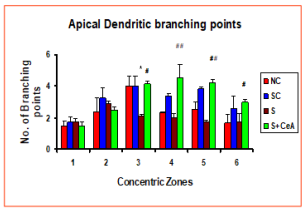
Results

There was significant decrease in the number of dendritic spines, dendritic branching points and dendritic intersections in the hippocampal CA3 neurons in group (iii) both in experiment I and experiment II even after 30 days after rehabilitation. However, there was significant increase in group (iv) which was subjected to restraint stress and treated with CeA leaves extract both in experiment I and after 30 days of rehabilitation (Experiment II).

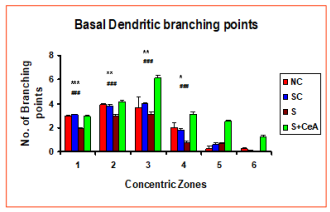
Experiment I

Effect of *Centella asiatica* leaves extract on restraint stress and stress + CeA treated groups

Apical and basal dendritic branching points at different concentric zones: Apical dendritic branching points were decreased in stressed group compared to normal and saline control groups beyond second concentric zones and basal branching branches in all concentric zones. In stressed+CeA treated group, the apical and basal branching points were increased in almost all concentric zones.



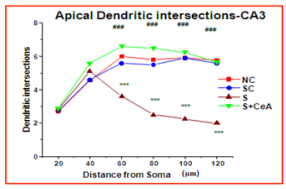
1a



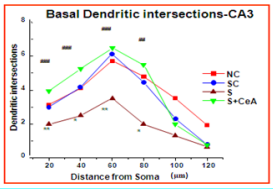
1b

Figure 1. 1a) Note there is a significant decrease in apical dendritic branching points in stressed group and which was increased in *Centella asiatica* leaves extract treated group. NC *vs* S - *p<0.05; S vs CeA - # p<0.05; ## p<0.01 (One way ANOVA, Bonferroni's Test).1b) Note there is a significant decrease in basal dendritic branching points in Stressed group and which was increased in *Centella asiatica* leaves extract Treated group. NC *vs* S.* p0.05, p<0.01,*** p<0.001; S vs CeA- #p<0.05, **###p<0.001 (One way ANOVA, Bonferroni's Test).

Apical and basal dendritic intersections: Apical and basal dendritic intersections were decreased in stressed group compared to normal and saline control groups. In stressed + CeA treated group, the apical and basal dendritic intersections were increased. Figures 2A and 2B



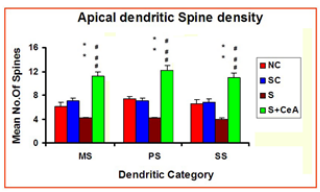
2a



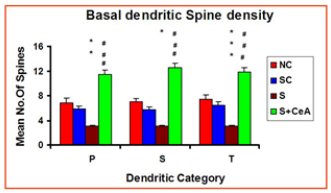
2b

Figure 2. 2a) Note there is a decrease in apical dendritic intersections in stressed group, which was increased in *Centella asiatica* leaves extract treated group. NC *vs* S - *** p<0.001; S *vs* CeA - ### p<0.001 (One way ANOVA, Bonferroni's Test). 2b) Note there is a decrease in basal dendritic intersections in stressed group, which was increased in *Centella asiatica leaves* extract treated group.NC *vs* S - p<0.05, **p<0.01; S *vs* CeA - ## p<0.01, ### p<0.001 (One way ANOVA, Bonferroni's Test).

Dendrites spine density: Dendritic spine density (100 µm length) on main shaft, primary and secondary branches of apical dendrites and primary, secondary and tertiary branches of basal dendrites were decreased in stressed group in all dendrites where as it was significantly increased both in apical and basal dendrites in stress + CeA treated group in all dendrites Figures 3A and 3B



3a



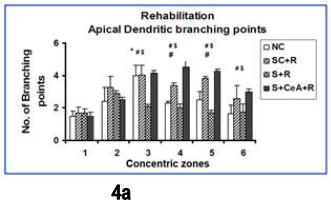
3b

Figure 3. 3a) Note there is a decrease in dendritic spine density in stressed group, which was increased in *Centella asiatica* leaf extract treated groups. NC *vs* S ** p<0.01, S *vs* CeA ### p<0.0001. 3b) Note there is a decrease in dendritic spine density in stressed group, which was increased in *Centella asiatica* leaf extract treated groups. NC *vs* S *p<0.05, ** p<0.01, ***p<0.001, S *vs* CeA ### p<0.001

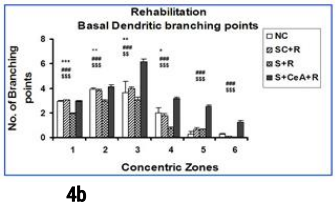
Experiment II

Effect of rehabilitation on restraint stress and stressed + CeA treated groups.

Apical and basal dendritic branching points at different concentric zones: Apical and basal dendritic branching points were decreased in stressed group at all concentric zones compared to normal and saline control groups. In stressed+CeA treated+rehabilitated group, the apical and basal branching points were increased in all concentric zones Figures 4A and 4B.



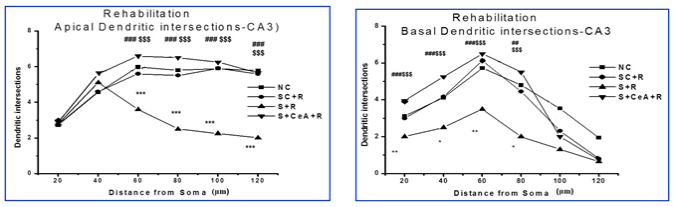
4a



4b

Figure 4. 4a) Note there is a decrease in apical dendritic branching points in stressed group even after 30days of cessation of the stress (rehabilitation period), and it remained increased in *Centella asiatica* leaf extract treated groups. NC *vs* S+R *p<0.05, S +R *vs* S+CeA+R # p<0.05, ##p<0.01. 4b) Note there is a decrease in basal dendritic branching points in stressed group even after 30days of cessation of the stress (rehabilitation period), and it remained increased in *Centella asiatica* leaf extract treated groups. NC *vs* S+R *p<0.05, ** p<0.01, *** p<0.001, S+R *vs* S+CeA+R # p<0.05, ### p<0.001.

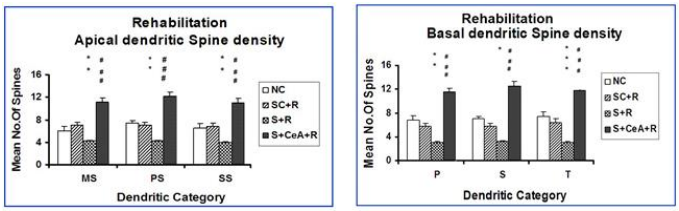
Apical and basal dendritic intersections at different concentric zones: Apical and basal dendritic intersections were decreased in stressed group at all concentric zones compared to normal and saline control groups. In stressed + CeA treated + rehabilitated group, the apical and basal dendritic intersections were increased in all concentric zones Figures 5A and 5B.



5a 5b

Figure 5. 5a) Note there is a decrease in apical dendritic intersections in stressed group even after rehabilitation, which remained increased in *Centella asiatica* leaf extract treated groups. NC *vs* S+R ***p<0.001, S+R *vs* S+CeA+R ### p<0.001. 5b) Note there is a decrease in basal dendritic intersections in stressed group even after rehabilitation, which remained increased in *Centella asiatica* leaf extract treated groups. NC *vs* S+R *p<0.05, **p<0.01, S+R vs S+CeA+R ## p, 0.01, ### p<0.001.

Dendrites spine density: Dendritic spine density (100 µm length) on main shaft, primary and secondary branches of apical dendrites and primary, secondary and tertiary branches of basal dendrites were decreased in stressed group where as it was significantly increased both in apical and basal dendrites. In stressed + CeA treated + rehabilitated group Figures 6A and 6B.



6a 6b

Figure 6. 6a) Note there is a decrease in apical dendritic spine density in stressed group even after rehabilitation, which remained increased in *Centella asiatica* leaf extract treated groups. NC *vs* S+R **p<0.01, S+R *vs* S+CeA+R ### p<0.001. 6b) Note there is a decrease in basal dendritic spine density in stressed group even after rehabilitation, which remained increased in *Centella asiatica* leaf extract treated groups. NC *vs* S+R **p<0.01, S+R *vs* S+CeA+R ### p<0.001.

Discussion

In the present study the effect of CeA leaves extract on hippocampal CA3 neurons in stressed mice was studied. Results showed an anti-stress effect of CeA and it reversed the stress induced neuronal atrophy in stressed + CeA extract treated group. Further rehabilitation experiment proved that the changes in the dendritic morphology of hippocampal CA3 neurons are of permanent nature.

In the present study, we have observed loss in dendritic arborization of hippocampal CA3 neurons in stressed group which may affect the functions of hippocampus. The mechanism of such dendritic atrophy may be due to:

Excitotoxicity

Excitotoxicity is a pathological condition resulting in neuronal death due to excessive release of excitatory neurotransmitter, glutamate from presynaptic nerve terminals into extracellular space. Excessive stimulation of glutamate receptors causes calcium influx which mediates glutamate excitotoxicity. Elevated glucocorticoid concentrations from the biological process pathways increase glutamate accumulation two times, in contrast to stress which causes four times increase in glutamate accumulation [15]. Restraint stress has been shown to increase glutamate uptake and release in the hippocampus and increased glutamate uptake was clearly seen after 30min of stress [16]. The major excitatory inputs to the hippocampus originate in the entorhinal area, an ipsilateral (perforant path) pathway activates the granule cells of the dentate gyrus, which in turn innervates the CA3 pyramidal neurons and glutamate is the transmitter involved in this pathway [17]. Entorhinal cortex lesioning protects stress induced atrophy of hippocampal CA3 neurons, which was claimed to be excitotoxic in nature [18].

Glucocorticoid toxicity

Glucocorticoid, the adrenal steroid hormone secreted during stress cause neuronal damage in the hippocampus as a result of excitotoxicity. Glucocorticoid selectively increase accumulation of extracellular excitatory amino acids in hippocampal cultures during excitotoxic insults like ischemia [19]. Glucocorticoid treatment for 21 days induces atrophy of hippocampal CA3 neurons which is prevented by steroid synthesis blocker, cyanoketone treatment [20].

Both stress and glucocorticoid increase glutamate concentrations in the hippocampal synapse [21].

Since hippocampal neurons have predominately more glucocorticoid receptors, binding of glucocorticoids to the receptors enhance calcium currents in hippocampus [22]. Glucocorticoids are known to increase the cytosolic calcium load in the hippocampus [23].

Glucocorticoids inhibit transcription of the calcium-ATPase pump, which plays a key role in extruding cytosolic calcium [24]. Both glucocorticoids and stress worsen calcium-dependent degenerative events such as cytoskeletal proteolysis and tau immunoreactivity [10].

Increased levels of calcium have been shown to disassemble microtubules and NMDA-receptor activation, calcium influx causes breakdown of MAP2 - a cytoskeletal phosphoprotein that regulates dynamic assembly of microtubules and also provide scaffolding for organelle distribution into the dendrites and restricts signal transduction apparatus in dendrites [25, 26]. This may lead to collapse and retraction of dendritic branches, since structural integrity of neuronal processes depends on the presence of stable microtubule.

Brain-Derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor, a major neurotrophic factor in the brain, is critical for the survival and function of neurons in the adult brain. However, recent evidence suggests that BDNF also plays a rapid and essential role in regulating synaptic plasticity, also modulates learning and memory after a stressful event. Stress and glucocorticoids are reported to decrease the expression of BDNF in the hippocampus and dentate gyrus. In the absence of BDNF the neurons undergo a process of programmed cell death or apoptosis. Corticosterone induces damage to cultured hippocampal neurons via reducing their BDNF synthesis and this is attenuated by exogenously added BDNF. Yet, BDNF alone is not sufficient to produce the changes observed after stress. BDNF along with glucocorticoids and other molecules facilitate both the morphological and molecular changes that occur.

Particularly changes in spine density and gene expression in response to stress [27, 28].

The probable mechanisms involved in increasing dendritic spine density, branching points and intersections (dendritic morphology) of hippocampal CA3 neurons and protection against stress induced neurodegeneration may be due to:

Formation of new spines could be because of unusual axon terminal sprouting followed by rearrangement of synapses on the existing dendrites after atrophy. Nora EG et al., in their experiment reported that several constituents of *Centella asiatica* can increase synaptogenesis and arborization in isolated hippocampal neurons (*In vitro*) but in the present case it was oral intubation of the CeA extract (In vivo) and it increased the dendritic arborization and dendritic spine density in CA3 hippocampal neurons [29].

The exact mechanism by which CeA improves dendritic morphology requires explanation. The spine formation and dendritic arborization involves a variety of biological pathways and processes like transcription factors, receptor-ligand interactions, various signaling pathways, cytoskeletal elements, Golgi outposts and endosomes contributes to the organization of dendrites in neurons [30].

CeA downregulates histone H3K9me3 which improves memory, promotes spine formation and increases BDNF levels in the hippocampus [31]. Histone H3K9 can turn the genes on by getting acetylated and silence them easily when methylated.

Centella asiatica activate ERK/RSK signaling pathway in neuroblastoma cells providing a possible molecular mechanism for memory enhancing property of CeA extract for the first time [32]. CeA significantly upregulated the level of activated ERK1/2 and Akt suggesting their involvement in the neurite outgrowth promoting activity [33, 34] but the exact molecular mechanisms are still restricted.

Conclusion

From the results of our present research study we conclude that, oral intubation of *Centella asiatica* leaves extract in restraint stressed albino mice leads to the following:

- Significantly increased the dendritic branching points and intersections in both apical and basal dendrites of hippocampal neurons.
- There was increase in dendritic spine density also in both apical and basal dendrites of hippocampal neurons.
- Rehabilitation for a period of 30 days in the normal laboratory conditions, failed to reverse the stress induced neuronal damage in the hippocampus.
- Rehabilitation for the same duration in the treated groups did not reverse the changes brought about by the plant extract suggesting the permanent nature of the changes.

Significance of the study

- This study proves the efficiency of these plant extracts as neuro stimulants and neuroprotective nature, protecting the neurons against the stress induced neuronal atrophy.
- These plant extracts can be used to treat the stress disorders.
- They can also be used to prevent age related, drug induced or spontaneous neurodegeneration.

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Cite this article: Hemamalini., *Centella asiatica* leaves extract causes permanent changes in the dendritic architecture of Hippocampal CA3 neurons in albino mice. J Neuro Neurophysiol 2024, 15 (1), 001-004.