Enhancement of Hippocampal CA3 Neuronal Dendritic Arborization by *Centella asiatica* (Linn) Fresh Leaf Extract Treatment in Adult Rats

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Background: *Centella asiatica* (CeA) is a creeper, growing in moist places in India and other Asian countries. Leaves of CeA are used for memory enhancement in the Ayurvedic system of medicine, an alternate system of medicine in India. In the present study, we investigated the role of CeA fresh leaf extract treatment on the dendritic morphology of hippocampal CA3 neurons, one of the regions concerned with learning and memory, in adult rats.

Methods: In the present study, adult rats (2.5 months old) were fed with 2, 4 and 6 mL/kg body weight of fresh leaf extract of CeA for 2, 4 and 6 weeks, respectively. After the treatment period, the rats were killed, brains were removed and hippocampal neurons were impregnated with silver nitrate (Golgi staining). Hippocampal CA3 neurons were traced using camera lucida, and dendritic branching points (a measure of dendritic arborization) and intersections (a measure of dendritic length) were quantified. These data were compared with those of age-matched control rats.

Results: The results showed a significant increase in the dendritic length (intersections) and dendritic branching points along the length of both apical and basal dendrites in rats treated with 6 mL/kg body weight/day of CeA for 6 weeks. However, the rats treated with 2 and 4 mL/kg body weight/day for 2 and 4 weeks did not show any significant change in hippocampal CA3 neuronal dendritic arborization.

Conclusion: We conclude that constituents present in *Centella asiatica* fresh leaf extract has neuronal dendritic growth-stimulating properties. [*J Chin Med Assoc* 2008;71(1):6–13]

Key Words: adult rats, *Centella asiatica,* dendritic branches, dendritic intersections, hippocampal CA3 neurons

Introduction

A number of plants, individually and in combination, are used in Ayurveda, an alternative system of medicine in India, for the treatment of a variety of diseases. *Medhya rasayana* is a group of such herbal medicines that are known to act on the central nervous system and have been claimed to improve mental ability.[1] These drugs mainly contain extracts from plants like *Centella asiatica*, *Acorus calamus*, *Jatamansi* and *Baccopa monnieri*.

C. asiatica is a herb that grows in damp places throughout India. It is used in Ayurvedic preparations either as a whole plant or leaves in fresh or extract form.[1] *C. asiatica* has been shown to be useful in improving learning and memory.[2–4] In addition, the plant is also used in mentally retarded children to improve general mental ability and in people suffering from cognitive disorders.[3,5–7] Though the fresh leaf juice (extract) of *C. asiatica* has been claimed to improve learning and memory in different clinical studies,[2,3,5,6] there is no evidence to show the effect of this plant extract on the brain regions involved in learning and memory, namely the hippocampus,[8–10] amygdala and limbic cortex. The cornu ammonis (CA) region, particularly the CA3 subregion of the hippocampus, is the key structure of the brain involved in learning and memory.[11–14]

We hypothesized that treatment with the fresh leaf juice of *C. asiatica* would bring about structural

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changes in the hippocampal CA3 pyramidal neurons of adult rats. In this investigation, we aimed to conduct the experiment in the same way as explained in the classic texts of Ayurveda,[1] i.e. without going for extraction, but using fresh leaf juice.

Methods

Rats

Adult Wistar rats (2.5 months old) of both sexes, bred and maintained in an institutional central animal research facility, were used in this experiment. The rats were fed with food and water *ad libitum* and maintained in a 12:12 hours cycle of dark and light. Room temperature was kept constant at 25°C. All experiments were carried out with prior approval from the institutional animal ethics committee. Only the minimum required number of rats was used, and rats were handled humanely.

Experimental groups

Rats were assigned into 2-, 4- and 6-week treatment groups. Rats in each of these groups were divided into 2 mL/kg, 4 mL/kg and 6 mL/kg body weight dose groups ($n=8$ for each dose). Each rat in the given dosage group was fed with the designated amount of fresh leaf extract of *C. asiatica* daily through gastric intubation for 2, 4 or 6 weeks. Along with these experimental groups, a normal control group and a vehicle (saline) control group ($n=8$ in both groups) were also maintained.

Extraction and administration of C. asiatica leaf juice

The plant, *C. asiatica*, was identified by Professor P. Venugopal Tantry of the Department of Botany, Vijaya College, Mulky, Karnataka, India. A voucher specimen number "525PP" was given and entered in the registry in the Department of Pharmocognosy, Manipal College of Pharmaceutical Sciences, Manipal, India. For the present experiment, we cultivated the plant in uniform soil conditions in order to maintain the same source of plant throughout the experiment.

Fresh, 15–20-day-old mature leaves of *C. asiatica* were collected in the morning. Fresh leaf juice was extracted from these leaves after washing, air-drying and homogenizing in a glass vessel and finally filtered through a sterile gauge cloth. Leaves were extracted maximally so that from a given weight of leaves, a known volume of juice was extracted (1.63 ± 0.15 mL of juice from 5 g of leaves, $n=6$). Since soil water was maintained uniform, we could extract the same volume of juice from the given weight of leaves on different days. Further, we established that the dry weight of a given volume (1 mL) of juice prepared on different days was the same (0.079 ± 0.01 g/mL of juice, $n=6$). The fresh leaf juice so obtained was fed to the rats as such, through a gastric tube, a capillary tube attached to a 1-mL hypodermic syringe. Since the volume of juice to be fed to individual rats was very small, the juice was blended with an appropriate volume of saline for convenient feeding. Control rats remained undisturbed in their home cage, and saline control rats were fed with a volume of saline that was equivalent to the volume of blended juice extract that their age-matched experimental rats received on each day.

Since standard extraction procedures, which involve boiling in water, ethyl alcohol or other organic solvents, may alter the structure of bioactive principles, we avoided using standard extraction protocols. Though there may have been variation in our daily preparations, such variation was expected to be minimal as leaves of equal maturation were collected from the same place on all days. The effects of minor daily variations, if any, would also be expected to be neutralized by the long period (2, 4 or 6 weeks) of treatment. It has been shown in a recent study that *C. asiatica* plant extract obtained from ethanol extraction is different from that by water extraction in its biological activity.[15]

Rapid Golgi staining procedure

At the end of the treatment period (2, 4 or 6 weeks), the rats were deeply anesthetized with anesthetic ether and killed, the brains were removed quickly, and the hippocampi dissected and fixed in rapid Golgi fixative. Tissue was processed for rapid Golgi staining as detailed previously.[16] Briefly, tissues were fixed for 5 days in Golgi fixative, and impregnated with 0.75% aqueous silver nitrate solution for 48 hours. Sledge microtome sections of 120 μm thickness were taken, dehydrated, cleared and mounted with Distrin plasticizer xylene mounting media.

Camera lucida tracing

Slides were coded prior to camera lucida tracing and quantification to avoid experimenter's bias. Decoding was done after completion of data collection and analysis. From each rat, 8–10 hippocampal CA3 neurons were traced using camera lucida, and their dendritic branching points and dendritic intersections were quantified. Neurons with minimal overlap of dendrites, heavily impregnated with silver nitrate and without truncate dendrites, were selected for tracing.

Table 1. Net body weight gain of rats treated with 2, 4 and 6 mL of *Centella asiatica* for 2, 4 and 6 weeks, respectively, and of age-matched control and saline-treated rats*

Group	$n^{\dagger}$	Body weight gain (g)		
		2 wk	4 wk	6 wk
Normal control	8	13.25 ± 1.11	32.19 ± 2.28	54.14 ± 20.51
Saline control	8	12.75 ± 0.93	31.34 ± 5.64	56.26 ± 4.52
Centella asiatica				
2 mL	8	13.98 ± 1.61	34.32 ± 7.25	59.82 ± 1.33
4 mL	8	15.02 ± 0.98	37.13 ± 6.66	55.56 ± 10.3
6 mL	8	14.83 ± 2.55	36.25 ± 4.96	68.66 ± 15.99

**Data are presented as mean ± standard deviation; †number of rats.*

Quantification of dendritic branching points and dendritic intersections (a measure of dendritic length)

The concentric circle method of Sholl[17] was used for dendritic quantification. On a transparent sheet, concentric circles were drawn. The radial distance between 2 adjacent concentric circles was 20 microns. For dendritic quantification (dendritic branching points and intersections), the sheet with concentric circles was placed on the camera lucida trace of the neuron in such a way that the center of the cell body of the neuron coincided with the center of the concentric circles. The number of branching points between the 2 successive concentric circles, i.e. within each successive 20 μm radial sphere, was counted. The dendritic intersection is the point where a dendrite intersects the given concentric circle. The dendritic intersections at each concentric circle were counted. Both branching points and intersections were counted up to a radial distance of 100 μm from the center of the soma. Mean number of dendritic branching points in each concentric zone/neuron and number of dendritic intersections at each concentric circle/neuron were calculated. This method of scoring was applied for both apical and basal dendritic quantification.

Data analysis

Data were analyzed using 1-way analysis of variance (ANOVA, nonparametric), followed by Dunnett's post test using GraphPad Prism version 3 (GraphPad Software Inc., San Diego, CA, USA).

Results

The rats treated with *C. asiatica* remained healthy throughout the treatment period. Moreover, they were more active than control rats, and learning and memory was significantly better in rats treated with higher doses for longer periods.[18] Briefly, the rats treated with the highest dose (6 mL) of *C. asiatica* performed better during spatial learning T-Maze tests, i.e. they showed an increased ($p<0.001$) number of alternations and decreased ($p<0.001$) percentage bias during spontaneous alternation test and increased ($p<0.001$) percentage of correct responses during rewarded alternation test. These rats also showed enhanced memory retention power, i.e. less ($p<0.001$) time spent in small compartment during the retention period of the passive avoidance test.[18] In addition to the behavioral changes, these rats showed better body weight gain than that of control and saline-treated rats (Table 1).

Hippocampal CA3 neuronal dendritic quantification

Hippocampal CA3 neuronal dendritic analyses in *C. asiatica* leaf extract treated rats showed significant increase in dendritic length and branching both in the apical and basal dendrites in the group who received the highest dose (6 mL/kg *C. asiatica*) when treated for 6 weeks. Figure 1 illustrates the dendritic arborization of hippocampal CA3 neurons in control rats and rats treated with 6 mL/kg *C. asiatica* leaf extract for 6 weeks. There was no difference in dendritic length and branching pattern between control and saline-treated rats, suggesting that daily handling of the rats (handling stress and vehicle) itself did not alter dendritic pattern. Since there was no significant difference in the dendritic length and branching between the control and vehicle groups, only comparisons between the control and experimental groups are detailed below and in the figures and table.

Results of 6 weeks of treatment

There was a significant increase in the dendritic intersections and branching points in the *C. asiatica* leaf extract treated rats at different radial distances from

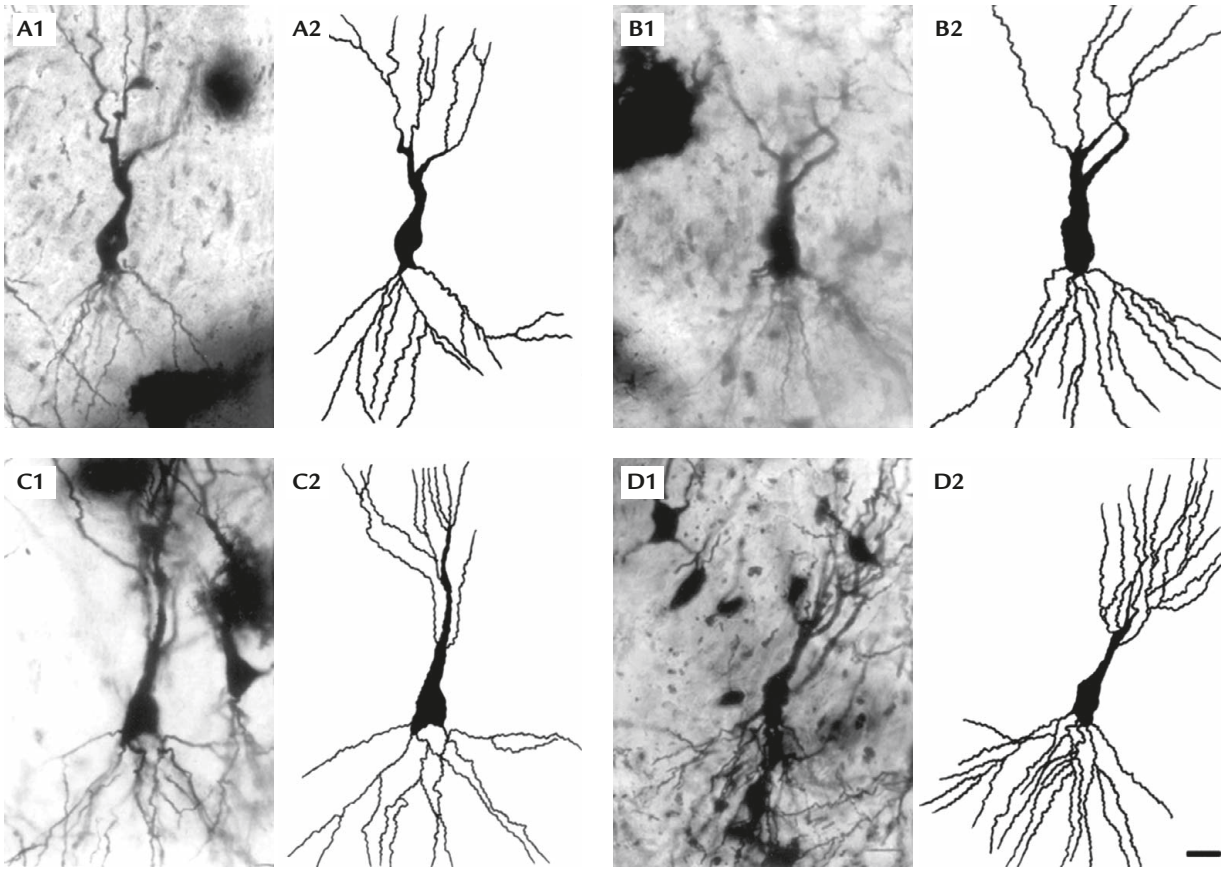

Figure 1. Representative photomicrographs (A1, B1, C1, D1) and camera lucida tracings (A2, B2, C2, D2) of Golgi-stained hippocampal CA3 neurons from control rats (A1, A2) and rats treated with *Centella asiatica* for 6 weeks at doses of 2 mL/kg (B1, B2), 4 mL/kg (C1, C2), and 6 mL/kg (D1, D2). Note the significant increase in dendritic length and branches in rats treated with 6 mL/kg of *C. asiatica* for 6 weeks (D1, D2). Scale bar = 20 μm.

the soma. Figure 1 illustrates photomicrographs and camera lucida tracings of normal controls (A1, A2) and 2, 4 and 6 mL of *C. asiatica* leaf extract treated rats (B1, B2, C1, C2, D1, D2, respectively) for 6 weeks. Note that these Golgi-stained (silver nitrate-impregnated) neurons have significantly increased number of dendrites extending from a distance of 20 μm to 100 μm from the soma in the rats treated with 6 mL/kg of *C. asiatica* leaf extract (D1, D2).

Apical dendritic intersections: The *C. asiatica* 6 mL group showed a significant increase in dendritic intersections at 40 μm (47.69% increase), 60 μm (118.07% increase), 80 μm (66.39% increase) and 100 μm (72.05% increase) concentric circles. There were no significant changes in the dendritic intersections at any of the concentric circles in the 2 mL/kg and 4 mL/kg *C. asiatica* groups when compared to the normal control group (Figure 2).

Basal dendritic intersections: The *C. asiatica* 6 mL group showed a significant increase in the dendritic intersections at 40 μm (47.72% increase), 60 μm (60.45% increase), 80 μm (65.48% increase) and 100 μm (90.5% increase) concentric circles. There were no significant changes in the dendritic intersections at any of the concentric circles in the 2 mL/kg and 4 mL/kg *C. asiatica* groups when compared to the normal control group (Figure 3).

Apical dendritic branching points: The *C. asiatica* 6 mL group showed a significant increase in the dendritic branching points in the 20–40 μm, 40–60 μm, 60–80 μm and 80–100 μm concentric zones. The increase in the 2nd (20–40 μm) zone was 91.13%, in the 3rd (40–60 μm) zone was 138.75%, in the 4th (60–80 μm) zone was 106.2%, and in the 5th (80–100 μm) zone was 120.45%. However, no significant changes were observed in the dendritic branching points at any of the concentric zones in the 2 mL/kg and 4 mL/kg *C. asiatica* groups (Figure 4).

Basal dendritic branching points: The *C. asiatica* 6 mL group showed a significant increase in the dendritic branching points in the 20–40 μm, 40–60 μm and 60–80 μm concentric zones. The increase in the

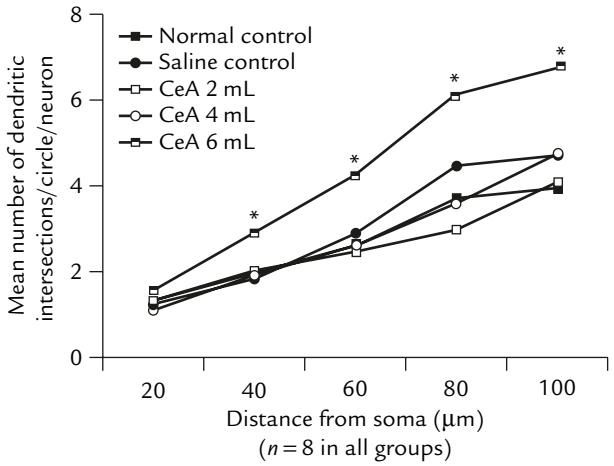

Figure 2. Apical dendritic intersections of hippocampal CA3 neurons in rats treated with 2, 4 and 6 mL/kg body weight of *Centella asiatica* (CeA) for 6 weeks, age-matched control and saline-treated rats. Each point represents the mean of 8–10 neurons from each rat (standard deviation not shown). *F* values are 4.61, 6.07, 7.88, 15.00 and 9.58 at distances of 20, 40, 60, 80 and 100 μm from the soma, respectively. Note the significant increase in dendritic intersections in rats treated with 6 mL/kg CeA. *$p < 0.01$, normal control *vs*. 6 mL/kg CeA (nonparametric 1-way ANOVA, Dunnett's test).

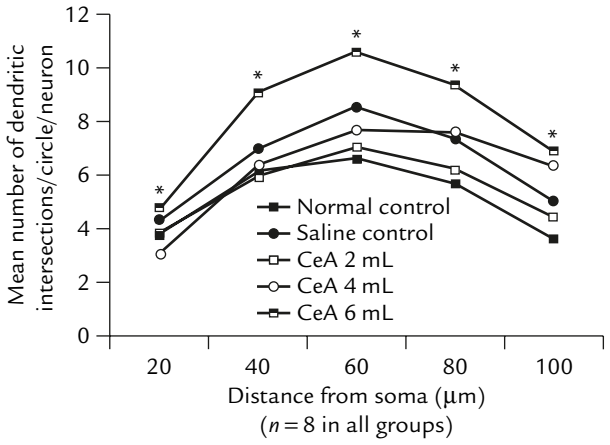

Figure 3. Basal dendritic intersections of hippocampal CA3 neurons in rats treated with 2, 4 and 6 mL/kg body weight of *Centella asiatica* (CeA) for 6 weeks, age-matched control and saline-treated rats. Each point represents the mean of 8–10 neurons from each rat (standard deviation not shown). *F* values are 8.39, 15.18, 13.16, 5.84 and 4.65 at distances of 20, 40, 60, 80 and 100 μm from the soma, respectively. Note the significant increase in dendritic intersections in rats treated with 6 mL/kg CeA. *$p < 0.01$, normal control *vs*. 6 mL/kg CeA (nonparametric 1-way ANOVA, Dunnett's test).

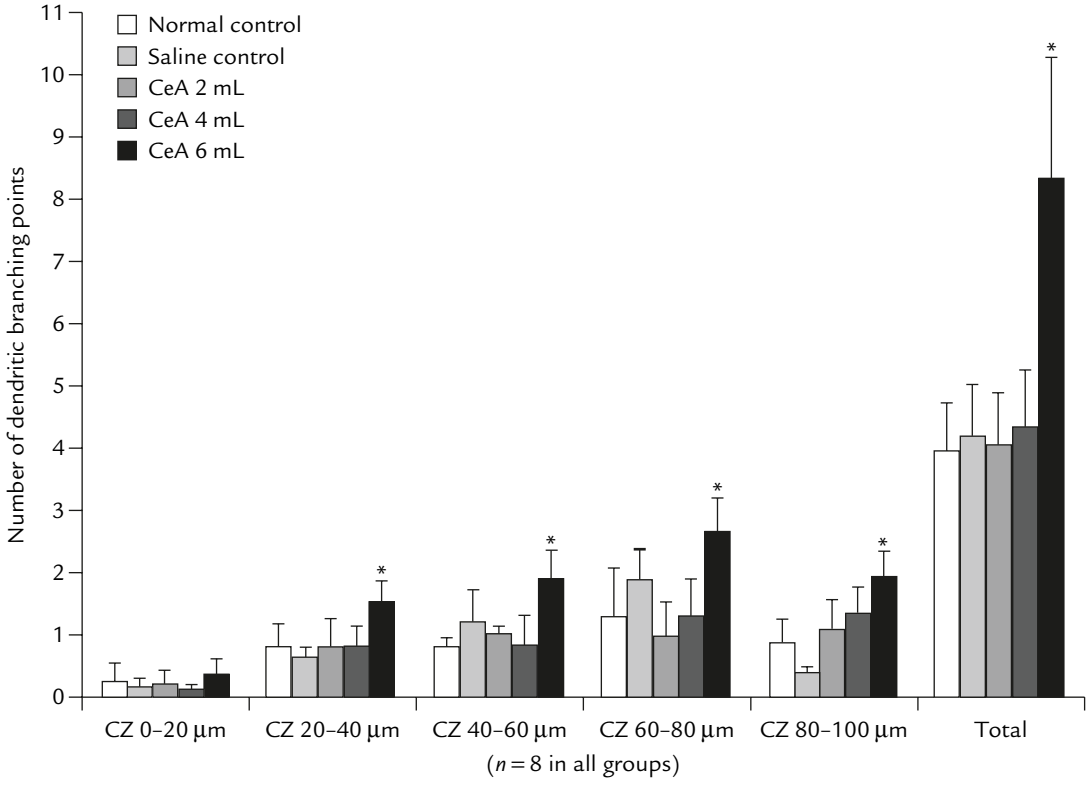

Figure 4. Apical dendritic branching points in the hippocampal CA3 neurons of rats treated with 2, 4 and 6 mL/kg body weight of *Centella asiatica* (CeA) for 6 weeks, age-matched control and saline-treated rats at different concentric zones (CZ) and total number of branching points. Each value represents the mean ± standard deviation of 8–10 neurons from each rat. *F* values are 1.60, 8.71, 11.39, 9.98, 18.22 and 21.66 at 0–20, 20–40, 40–60, 60–80, 80–100 μm CZ and total number of branching points, respectively. Note the significant increase in dendritic branching points in the rats treated with 6 mL/kg CeA compared to control rats. *$p < 0.01$, normal control *vs*. 6 mL/kg CeA (nonparametric 1-way ANOVA, Dunnett's test).

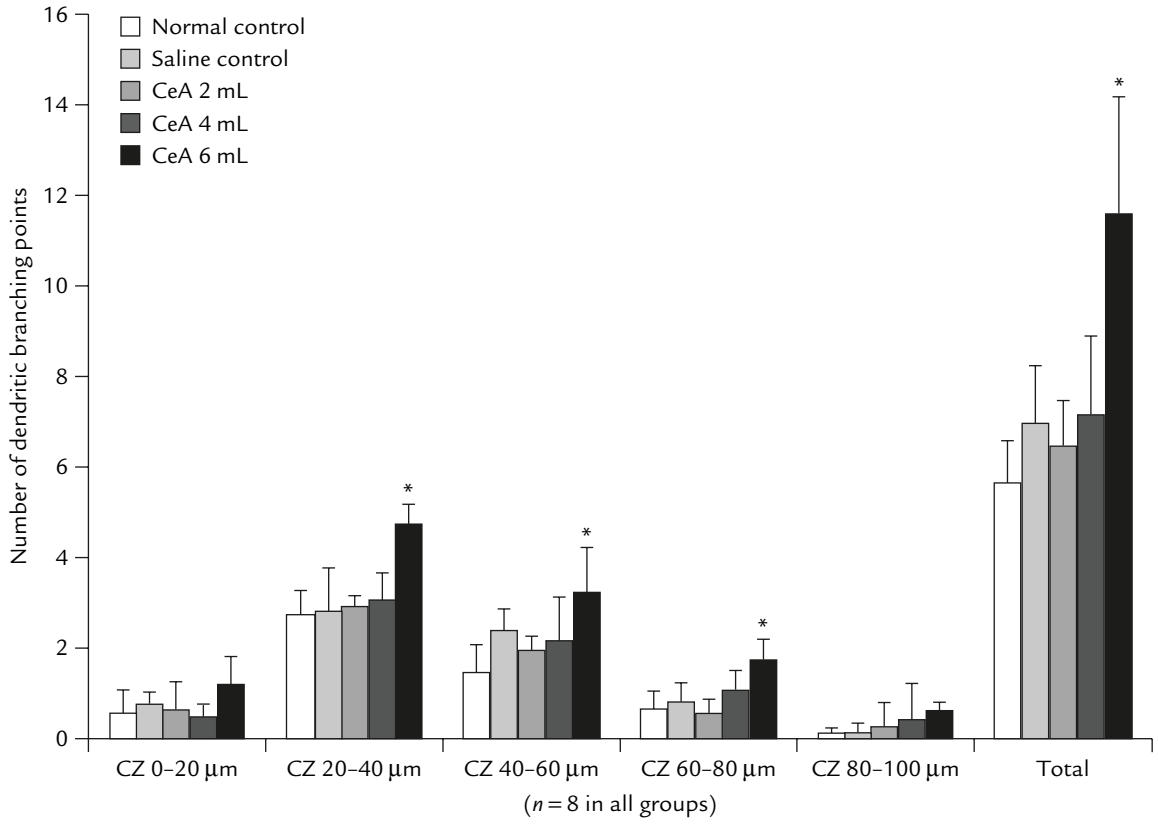

Figure 5. Basal dendritic branching points in the hippocampal CA3 neurons of rats treated with 2, 4 and 6 mL/kg body weight of *Centella asiatica* (CeA) for 6 weeks, age-matched control and saline-treated rats at different concentric zones (CZ) and total number of branching points. Each value represents mean ± standard deviation of 8–10 neurons from each rat. *F* values are 2.36, 14.39, 6.40, 10.25, 1.41 and 17.01 at 0–20, 20–40, 40–60, 60–80, 80–100 µm CZ and total number of branching points, respectively. Note the significant increase in dendritic branching points in the rats treated with 6 mL/kg CeA compared to control rats. $*p < 0.01$, normal control *vs*. 6 mL/kg CeA (nonparametric 1-way ANOVA, Dunnett's test).

2nd (20–40 µm) zone was 72.26%, in the 3rd (40–60 µm) zone was 122.75% and in the 4th (60–80 µm) zone was 106.2%. However, no significant change was observed in the dendritic branching points at any of the concentric zones in the 2 mL/kg and 4 mL/kg *C. asiatica* groups (Figure 5).

Results of 4 weeks of treatment

There was no significant change in both apical and basal dendritic intersections as well as branching points of hippocampal CA3 neurons in rats treated with *C. asiatica* for 4 weeks in any of the dose (2, 4 and 6 mL/kg) groups (data not shown).

Results of 2 weeks of treatment

There was no significant change in both apical and basal dendritic intersections as well as branching points of hippocampal CA3 neurons in rats treated with *C. asiatica* for 2 weeks in any of the dose (2, 4 and 6 mL/kg) groups (data not shown).

Discussion

C. asiatica fresh leaf extract treatment (6 mL/kg body weight) for 6 weeks showed 47–118% increase in apical and 47–90% increase in basal dendritic intersections in the outer concentric zones. Similarly, there was a total of 111% increase in the apical (Figure 4) and 105% increase in the basal (Figure 5) dendritic branching points in the 6 mL/kg body weight dose group. However, the lower dose groups (4 mL/kg and 2 mL/kg) and shorter duration (2 and 4 weeks) groups failed to show significant increase in the dendritic intersections and branching points in both apical and basal dendrites.

There are several reports in the literature indicating the dendritic modifications brought about by various factors. We have earlier reported enhancement of hippocampal CA3 neuronal dendritic arborization by *C. asiatica* fresh leaf extract treatment in neonatal rats.[19] Repeated exposure to enriched environments has been shown to increase the density of spines and

dendritic complexity in certain brain structures.[20] After induction of long-lasting functional enhancement of synapses in the CA1 area, new spines appear on post-synaptic dendrites, whereas in control regions on the same dendrites or in slices where long-term potentiation is blocked, no significant spine growth occurs.[21] These reports suggest that dendritic structural reorganization is possible at these regions.

The dendrites of hippocampal CA3 neurons receive extrinsic and intrinsic inputs from the different parts of the limbic system. The extrinsic afferents are mainly from the entorhinal cortex, septal area, mamillary body, and nuclei of the brain stem.[22,23] The CA3 neurons also receive a significant number of intrinsic afferents from the axons of dentate granule cells and the contralateral CA3 regions.[24,25] Increase in the dendritic length of the dendritic branches in hippocampal CA3 neurons may result in alterations in synaptic connectivity. It may result in alteration in learning and memory. Indeed, we and others have reported improved learning and memory in neonatal and adult rats treated with *C. asiatica*.[26,27]

On the other hand, increased dendrites in the hippocampal CA3 region may make it an epileptic locus. In the present study, the *C. asiatica* treated rats were observed 6 hours a day, 3 days a week for about a month for seizure activity, and no epileptic episodes were observed. Observation round the clock using video monitoring might have displayed any such epileptic episodes. However, there might have been electrographic seizures in these rats in the absence of behavioral seizures.

Increased dendritic intersections and branching of hippocampal CA3 neurons in animals treated with 6 mL of *C. asiatica* a day for 6 weeks only in the present study suggests that this dose of plant extract is effective for inducing structural changes in these neurons. Lower doses of *C. asiatica* fresh leaf extract and shorter duration of treatment failed to induce these structural changes. The increased numbers of dendritic intersections and branches of hippocampal CA3 neurons may have profound effects on behavior because of the additional dendrites that are available on these neurons for the formation of new synapses. It is obvious from the results that a significant number of additional dendrites are formed closer to the soma of neurons (20–40 μm zone). This means that new synapses can be formed closer to the soma of the neurons, resulting in more rapid and effective conduction of impulses.

From the present study, it may be concluded that administration of fresh leaf extracts of *C. asiatica* for a longer period will result in dendritic growth in the hippocampal CA3 neurons in adult rats.

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