eISSN 1303-1775 • pISSN 1303-1783

Neuroanatomy (2005) 4: 18–23

Original Article

Centella asiatica (linn) induced behavioural changes during growth spurt period in neonatal rats

Published online 29 July, 2005 © http://www.neuroanatomy.org

K. G. Mohandas RAO [1] ✦
S. Muddanna RAO [2]
S. Gurumadhva RAO [3]

Departments of Anatomy [1] and Pharmacology [3], Meleka Manipal Medical College, Manipal—India;
Department of Anatomy [2], Kasturba Medical College, Manipal—India.

✦ Dr. K. G. Mohandas Rao
Assistant Professor in Anatomy,
Meleka Manipal Medical College,
576 104 Manipal—INDIA
☎ 91-820-257 12 01 (Extn:22519)
91-820-257 19 05
✉ babbarao@yahoo.com

Received 19 April 2005; accepted 28 July 2005

ABSTRACT

Neonatal rat pups (7 days old) were given different doses of fresh leaf juice of *Centella asiatica* (CeA) orally for different periods of time. These rats were then subjected to spatial learning (T- Maze) and passive avoidance tests along with the age matched normal and saline control rats. The results showed improvement in spatial learning performance and enhanced memory retention in neonatal rats treated with higher doses. These results indicate that treatment with CeA fresh leaf juice during growth spurt period of neonatal rats enhances memory retention. ***Neuroanatomy; 2005; 4: 18–23.***

Key words [*centella asiatica*] [growth] [spatial learning] [passive avoidance] [memory]

Introduction

A characteristic feature of animals and particularly of humans is the ability to alter their behaviour on the basis of experience or learning. Learning is an acquisition and storage of information as a consequence of experience. Memory is a relatively permanent storage form of the learned information [1]. The hippocampus and amygdala are two important regions involved in learning and memory.

In the ayurvedic system of medicine "Medhya drugs" are a group of medicines known to act on the nervous system. In the texts of Ayurveda, many medhya drugs have been claimed to improve mental ability [2]. Some of the drugs, which act on the nervous system, include *Bacopa monnieri*, Ashwagandha (*Withania somnifera*), Jyotishmati (*Celastrus panniculatus*) Shankapushpi (*Clitoria ternatea*), Jatamansi (*Nardostachys jatamansi*), Vacha (*Acorus calamus*) and Mandukaparni (Brahmi, *Centella asiatica*) [3–6].

Among these, *Centella asiatica* (CeA) is a herb growing in wet and marshy places throughout the country. It has been used in ayurvedic preparations either in the fresh or in the extract form [2]. *Centella asiatica* is shown to be very useful in improving learning and memory [4–6]. It is also used as a brain tonic for promoting brain growth and improving memory [7]. In addition, the plant is also used in mentally retarded children to improve general mental ability [5, 8–10].

Though the fresh juice of CeA has been claimed to improve learning and memory in different clinical studies, there are no direct neurological studies to show the action of fresh leaf juice of this plant on improvement of behaviour especially learning and memory in neonatal rats.

Thus this study was designed to find the effect of CeA fresh leaf juice treatment on learning and memory in neonatal rats. This experiment was carried out on neonatal rats, since, active brain growth occurs in them during pre and post-weaning period (growth spurt period) [11].

Materials and Methods

Animals and experimental groups

7 days old Wistar rats of both sexes maintained under 12 hours dark and 12 hours light cycle, provided with food and water ad libitum were used in the experiments.

Rat pups were divided into three major groups: 1) two weeks, 2) four weeks, 3) six weeks treatment groups. In each of these groups there were subgroups:

a. Normal control (NC): These animals remained undisturbed in their home cage till other groups completed their saline/CeA fresh leaf juice treatment,

b. Saline control (SC): These animals received equivolume of saline,

c. 2ml/kg CeA group: These animals received 2ml/kg CeA fresh leaf juice every day,

d. 4ml/kg CeA group: These animals received 4ml/kg CeA fresh leaf juice every day,

e. 6ml/kg CeA group: These animals received 6ml/kg CeA fresh leaf juice every day.

The experiments were carried out after the approval from the animal ethical committee.

Extraction and administration of *Centella asiatica* leaf juice

The plant, CeA was identified by Mr. P. Venugopal Tantry, Professor of Botany, Department of Botany, Vijaya College, Mulky, Karnataka, India and has been entered in and given the voucher specimen number "525PP" by the department of Pharmacognosy, Manipal College of pharmaceutical Sciences, Manipal, India. These plants were specially grown in uniform soil and water conditions. Fresh leaves of CeA were collected in the morning. Care was taken to collect the leaves of uniform growth (15–20 days old). After washing, air drying and homogenizing by grinding, the juice was extracted by squeezing the paste like homogenate using a piece of clean cloth. The fresh juice so obtained was administered as such or after appropriate dilution with saline by gastric intubation, using a capillary tube attached to a tuberculin syringe. The volume of juice to be given to the individual rat was calculated based on their body weight.

Behavioural tests

Following treatment, all the groups (NC, SC and CeA) of rats were subjected to behavioural tests. The behavioural tests included, 1) spatial learning (T- Maze) test and 2) passive avoidance test.

Spatial learning (T–maze) tests

The purpose of this test was to assess the spatial learning ability of the rats. This test included spontaneous alternation and rewarded alternation tests.

The wooden T–maze apparatus consisted a stem (35x12cm), a choice area (15x12 cm) and two arms (35x12cm). The start box (15x12 cm) was located at the beginning of the stem. The goal areas were at the ends of the two arms (each 15x12 cm) containing the food well. The stem and start box were separated by a sliding door. A cloth curtain separated the arm and goal areas. The height of the sidewall of the apparatus was about 40 cm. The apparatus was kept in a sound attenuated normally lit room.

Spontaneous alternation test [12]. Two days prior to the starting of the test, the rats were deprived of food in order to motivate them for the food reward. Subsequently, the food was restricted so that the animal's body weight was maintained at 85% of pre-test weight. This was followed by orientation, which was done to familiarize the rats with the T–maze. During orientation, the rats subjected for food restriction were placed in the start box for sixty seconds. The sliding door was then opened to allow the rat to explore the T–maze for thirty minutes, and to eat fifteen pellets (10 mg each) in each goal area. After thirty minutes the rat was returned to the start box. This procedure was carried out for two consecutive days for all rats of the group.

After the orientation, six trials were given daily for the following four days. In each trial, the rat was first placed in the start box. By opening the sliding door it was allowed to enter into the stem and allowed to choose any one of the arms. A rat was considered to have entered into a particular arm only when it entered that arm with all its limbs. Once the rat ate the pellet in the goal area of that arm, it was replaced back in the start box for the next trial. The intertrial interval was one minute.

In each trial, the arm chosen by the rat was noted. At the end of four days i.e. twenty-four trials, the total number of alternations were also noted. The percentage bias was calculated for each rat using the following formula.

$$\text{Percentage bias} = \frac{\text{Total number of choices of more frequently chosen side} \times 100}{\text{Total number of trials}}$$

More number of alternations and less percentage bias was considered as an index for improved learning ability.

Rewarded alternation test [2]. This test was started on the day after the completion of spontaneous alternation test. During this test, six trials per day were conducted for four days. Each trial had two runs namely, a forced run and a choice run. In the forced run, the animal was forced to one of the arms by blocking the other arm and was allowed to consume the pellet in the goal area. Once the animal ate the pellet in the goal area, it was placed back in the start box for a choice run. In the choice run, the goal area of the forced arm was kept empty and pellets were placed in the goal area of the opposite arm. But both the arms were kept free for the rat to choose. Between each forced run and the choice run, a gap of one minute was given. Similarly there was a gap of one minute between the two trials again. The sequence of the forced arm was predetermined and was same for all the rats for a given day. On subsequent days it was alternatively changed. For example on the first day of the test, if the animals were forced to the right arm of the T–maze, on the second day they were forced to enter the left arm. On the third day, again forced to the right arm and on the fourth day to the left arm. During the choice run, if the rat entered the arm opposite to the forced arm, then that response was considered as "correct response". If it entered the same arm to which it was forced during forced run, it was considered as "wrong response".

Percentage of correct responses was calculated for each rat by using the following formula.

$$\text{Percentage of correct responses} = \frac{\text{Total number of correct responses} \times 100}{\text{Total number of trials}}$$

Increase in percentage of correct response was considered as an index of improved learning and memory.

Passive avoidance test (Modified from Bures et al. [13])

The passive avoidance apparatus was fabricated locally. It had two compartments, a rectangular larger compartment with a 50x50 cm grid floor and wooden

walls of 35 cm height. It had a roof, which could be opened or closed. In the centre, one of the walls had a 6x6 cm opening connecting the larger compartment to a dark smaller compartment. The smaller compartment had 15x15 cm electrifiable grid connected to a constant current stimulator, wooden walls of 15 cm height and a ceiling, which could be opened or closed. The connection between the two compartments could be closed with a sliding door. The larger compartment was illuminated with a 100 W bulb placed 150 cm above the centre.

The experiment included three parts, 1) exploration test, 2) an aversive stimulation and learning (passive avoidance acquisition), and 3) retention test.

During *exploration test* each rat was kept in the centre of the larger compartment facing away from the entrance to the dark smaller compartment. The door between the two compartments was kept open. The rat was allowed to explore the apparatus (both larger and smaller compartments) for 3 minutes. In each trial, the total time spent by the animal in the smaller compartment was noted. At the end of the trial, the rat was replaced in the home cage, where it remained during inter-trial interval of five minutes.

After the last exploration trial, the rat was forced into the smaller compartment and the sliding door between the two compartments of the apparatus was closed. Three strong foot shocks (50 Hz, 1.5 mA, 1 sec duration) were given at approximately five-second intervals. The ceiling was then opened and the rat was returned to its home cage.

Retention test was carried out after twenty-four hours of acquisition test. The rat was kept in the centre of the larger compartment facing away from the entrance to the smaller compartment. The sliding door between the two compartments was kept open. The rat was allowed to explore the apparatus for three minutes. After three minutes the rat was kept back in the home cage. With a gap of five minutes the trial was repeated for three times. In each trial, the time spent by the rat in the smaller compartment was noted.

Decrease in the time spent in the smaller compartment during retention test was considered as good memory retention performance

Data analysis. Data was analyzed using analysis of variance (ANOVA) followed by Bonferroni's test (post-test) using Graph Pad In Stat (GPIS) software, version 1.13.

Results

Spatial learning (T-Maze tests)

Table-1 shows results of the T-Maze tests. In 2 weeks treatment group, during spontaneous alternation test, animals treated with 2ml of CeA fresh leaf juice did not show any significant difference in their performance. However, animals treated with higher doses of CeA fresh leaf juice (4 and 6 ml) showed significantly higher number of alternations when compared to normal control group of rats (10.1 ± 3.66 in normal control vs. 17.85 ± 2.34 in CeA 4 ml group, P< 0.01 and 17.95 ± 3.0 in CeA 6 ml, P<0.01).

Similarly, rats treated with higher doses (4 and 6 ml) of CeA showed significantly lesser percentage bias in comparison with normal control rats (69.59 ± 13.32 in normal control group vs. 51.18 ± 2.02 in CeA 4 ml group, P<0.05 and 50.56 ± 2.87 in CeA 6 ml group, P<0.05).

During rewarded alternation test also, only rats treated with higher doses (4 and 6 ml) of CeA fresh leaf juice showed a significant increase in the percentage of correct response when compared to the normal control group rats (63.68 ± 19.79 in normal control vs. 87.49 ± 9.62 in CeA 4 ml group, P< 0.01 and 90.47 ± 6.68 in CeA 6 ml group, P<0.01).

In 4 weeks treatment group, during spontaneous alternation test, the animals treated with 2, 4 and 6ml of CeA fresh leaf juice showed significantly higher number of alternations when compared to the normal control group of rats (12.62 ± 2.13 in normal control vs. 15.85 ± 0.89 in CeA 2 ml group, P<0.05, 19.0 ± 0.70 in CeA 4 ml group, P<0.001 and 16.37 ± 2.13 in CeA 6 ml group, P<0.01).

Table 1. Results of spatial learning (T-maze) tests

Groups	n	2 weeks treatment group			4 weeks treatment group			6 weeks treatment group		
		Spont. alt. test	% Bias	Rew. alt. test	Spont. alt. test	% Bias	Rew. alt. test	Spont. alt. test	% Bias	Rew. alt. test
		No. of alternations		% of correct response	No. of alternations		% of correct response	No. of alternations		% of correct response
Normal control (NC)	8	10.1±3.66	69.59±13.32	63.68±19.79	12.62±2.13	66.24±5.89	69.78±16.02	12.0±2.88	69.48±4.64	65.1±5.29
Saline control (SC)	8	15.0±1.00	57.49±4.56	76.66±11.25	13.37±1.76	56.24±4.45	76.03±9.38	15.16±2.31	64.85±5.52	66.1±6.26
CeA-2ml	8	14.71±2.62	55.35±2.03	82.13±8.9	15.85$^{\#}$±0.89	50.14$^{\#\#}$±3.96	92.85$^{\#}$±9.22	18.42$^{\#\#}$±2.69	56.51$^{\#\#}$±5.8	93.42$^{\#\#\#}$±5.3
CeA-4ml	8	17.85**±2.34	51.18*±2.02	87.49**±9.62	19.0***±0.7	52.49***±2.27	92.49*±15.43	19.5***±2.07	55.2**±6.58	95.83***±5.89
CeA-6ml	8	17.95$^{\$\$}$±3.0	50.56$^{\$}$±2.87	90.47$^{\$\$}$±6.68	16.37$^{\$\$}$±2.13	50.12$^{\$\$\$}$±4.31	90.14$^{\$}$±9.22	18.62$^{\$\$\$}$±2.06	55.2$^{\$\$}$±4.85	88.01$^{\$\$\$}$±10.55

Each value represents Mean±SD. *(**NC vs. CeA 2ml:** # P< 0.05, ## P<0.01, ### P<0.001; **NC vs. CeA 4ml:** * P<0.05, ** P<0.01, *** P<0.001; **NC vs. CeA 6ml:** $ P<0.05, $$ P<0.01, $$$ P<0.001; **CeA:** Centella asiatica; **n:** Number of rats)*

Similarly, the rats treated with 2, 4 and 6 ml of CeA fresh leaf juice showed significantly lesser percentage bias in comparison with the normal control group of rats (66.24 ± 5.89 in normal control vs. 50.14 ± 3.96 in CeA 2 ml group, P<0.01, 52.49 ± 2.27 in CeA 4 ml group, P<0.001 and 50.12 ± 4.31 in CeA 6 ml group, P<0.001).

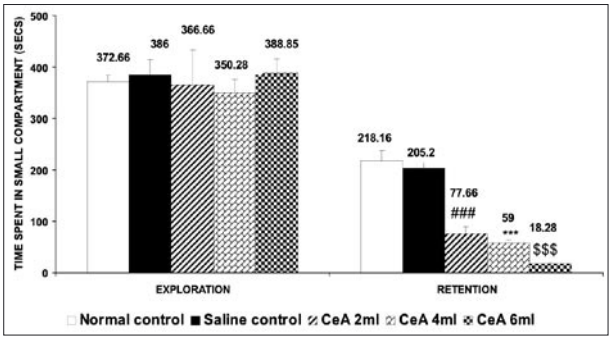

Figure 1. Graph showing the time spent in small compartment in 2 weeks treatment group. *(Normal control (n= 8), Saline control (n= 8), CeA 2 ml (n= 8), CeA 4 ml (n= 8), CeA 6 ml (n= 8). Each bar represents Mean + SD. NC vs. CeA 2 ml: ### P<0.001; NC vs. CeA 4 ml: *** P<0.001; NC vs. CeA 6 ml: $$$ P<0.001)*

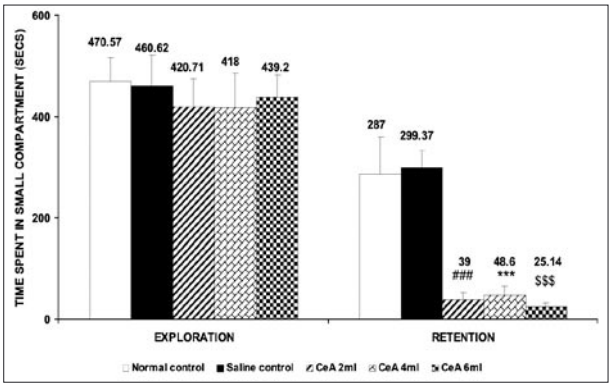

Figure 2. Graph showing the time spent in small compartment in 4 weeks treatment group. *(Normal control (n= 8), Saline control (n= 8), CeA 2 ml (n= 8), CeA 4 ml (n= 8), CeA 6 ml (n= 8). Each bar represents Mean + SD. NC vs. CeA 2ml: ### P<0.001; NC vs. CeA 4 ml: *** P<0.001; NC vs. CeA 6 ml: $$$ P<0.001)*

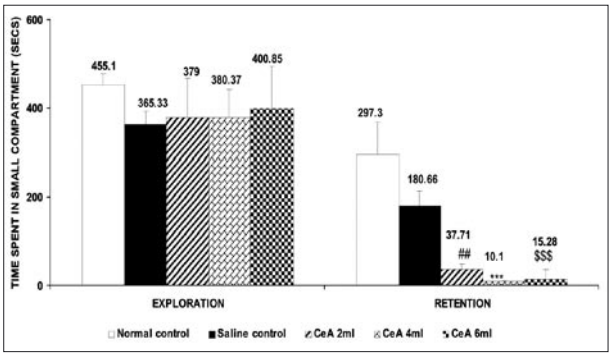

Figure 3. Graph showing the time spent in small compartment in 6 weeks treatment group. *(Normal control (n= 8), Saline control (n= 8), CeA 2ml (n= 8), CeA 4ml (n= 8), CeA 6ml (n= 8). Each bar represents Mean + SD. NC vs. CeA 2 ml: ### P< 0.001; NC vs. CeA 4 ml: *** P<0.001; NC vs. CeA 6 ml: $$$ P< 0.001)*

During rewarded alternation test, rats treated with three different doses (2, 4 and 6 ml) of CeA fresh leaf juice showed a significant increase in the percentage of correct response when compared to the normal control group rats (69.78 ± 16.02 in normal control vs. 92.85 ± 9.22 in CeA 2 ml group, P<0.05, 92.49 ± 15.43 in CeA 4 ml group, P<0.05 and 90.14 ± 9.22 in CeA 6 ml group, P<0.05).

In 6 weeks treatment group, during spontaneous alternation, test animals treated with all the 3 doses (2, 4 and 6 ml) of CeA fresh leaf juice showed significantly higher number of alternations when compared to normal control group of rats (12.0 ± 2.88 in normal control vs. 18.42 ± 2.69 in CeA 2 ml group, P< 0.01, 19.5 ± 2.07 in CeA 4 ml group, P< 0.001 and 18.62 ± 2.06 in CeA 6 ml group, P<0.001).

However, all the three groups of rats treated with CeA (2, 4 and 6 ml) showed significantly lesser percentage bias in comparison with normal control group (69.48 ± 4.64 in normal control vs. 56.51 ± 5.8 in CeA 2 ml group, P<0.01, 55.2 ± 6.58 in CeA 4 ml group, P<0.01 and 55.2 ± 4.85 in CeA 6 ml group, P<0.01).

During rewarded alternation test, rats treated with all the three different doses (2, 4 and 6 ml) of CeA fresh leaf juice showed a significant increase in the percentage of correct response when compared to normal control group (65.10 ± 5.29 in normal control vs. 93.42 ± 5.3 in CeA 2 ml group, P<0.001, 95.83 ± 5.89 in CeA 4 ml group, P<0.001 and 88.01 ± 10.55 in CeA 6 ml group, P<0.001).

Passive avoidance test

Results of passive avoidance exploration and retention performance are shown in figures 1, 2 and 3. All the CeA treatment groups showed good memory retention.

In 2 weeks treatment group (Figure 1) during exploration, there was no significant difference between animals treated with the CeA fresh leaf juice (2, 4 and 6 ml) and normal control animals in total time spent in small compartment. However, during retention test, it was seen that animals treated with CeA fresh leaf juice spent significantly less time in the smaller compartment (218.16 ± 20.94 sec in normal control vs. 77.66 ± 12.35 sec in CeA 2 ml group, P<0.001, 59.0 ± 5.28 sec in CeA 4 ml group, P<0.001 and 18.28 ± 2.88 sec in CeA 6 ml group, P<0.001).

In 4 weeks treatment group (Figure 2) during exploration, there was no significant difference between the animals treated with CeA fresh leaf juice (2, 4 and 6 ml) and the normal control animals in total time spent in small compartment. However, during retention test, it was observed that the animals treated with CeA fresh leaf juice spent significantly less time in the small compartment (287.0 ± 74.35 sec in normal control vs. 39.0 ± 14.24 sec in CeA 2 ml group, P<0.001, 48.6 ± 18.95 sec in CeA 4 ml group, P<0.001 and 25.14 ± 18.77 sec in CeA 6 ml group, P<0.001).

In 6 weeks treatment (Figure 3) group during exploration, there was no significant difference between the animals treated with CeA fresh leaf juice (2, 4 and 6 ml) and the normal control animals in total time spent in small compartment. However, during the retention test, animals

of all the three groups treated with CeA fresh leaf juice spent significantly less time in the small compartment (297.3 ± 71.94 sec in normal control vs. 37.71 ± 12.31 sec in CeA 2 ml group, P<0.01, 10.1 ± 1.92 sec in CeA 4 ml group, P<0.001 and 15.28 ± 22.69 sec in CeA 6 ml group, P<0.001).

Discussion

In the present study the results of T-Maze tests of rats treated with lower doses of CeA (2 ml) for shorter duration (2 weeks) were not significantly different than the normal rats. However animals of higher dose groups (4 and 6 ml) showed significant improvement in the learning behaviour even in shorter (2 weeks) duration of treatment. Rats when treated for longer duration (4 and 6 weeks) showed significant improvement in the learning behaviour in all (2, 4 and 6 ml) dose groups. In the passive avoidance tests, there was no significant change in behaviour during exploration. However, during retention test, animals of all the three dose groups (2, 4 and 6 ml) spent less time in the smaller compartment suggesting improved memory retention. This enhanced memory retention was observed in the animals treated with CeA for 2, 4 and 6 weeks.

These results clearly indicate that oral administration of fresh leaf juice of CeA improved learning and memory in neonatal rats. This effect was marked in animals treated with higher doses of CeA. Use of CeA in preventing radiation induced behavioural changes during clinical radiotherapy has been reported before [14]. Asiatic acid, a triterpine of CeA is used in the treatment of dementia and as an enhancer of cognition. Three derivatives obtained from CeA are found to be efficacious in protecting neurons from oxidative damage caused by exposure to excess glutamate [15]. Aqueous extract of CeA has an enhancing effect on cognitive functions [16]. *Centella asiatica* is also reported to improve general mental ability and behavioural pattern in mentally retarded children [5, 8–10]. Nalini et al. [17] have reported the memory enhancing effect of aqueous extract of CeA in adult rats. But the results of present study is the first experimental evidence regarding the memory enhancing property of CeA fresh leaf juice during growth spurt period of rats.

Treatment with *Clitoria ternatea* root extract has been shown to enhance memory in neonatal rats [3]. The exposure to the new learning experiences [18], intracranial self stimulation [19], living in enriched environment [20–22] and environment within pyramid model [23] has been shown to alter the cytoarchitecture of hippocampus which is a part of the brain concerned with learning and memory. Similarly fresh leaf juice of CeA has been shown to improve dendritic arborisation of amygdala and hippocampus [24–27]. Improved learning behaviour and enhanced memory retention in the present study is probably because of the structural changes in these brain regions [18, 28, 29].

It has been observed that CeA treatment increases the level of neurotransmitter GABA that is known to act on hippocampus [30, 31]. Similarly CeA may also affect the biosynthesis of other neurotransmitters involved in learning and memory like Ach, noradrenaline, 5HT, dopamine [32–34].

However these morphological, neurophysiological and neurochemical changes need to be investigated.

We conclude by saying that oral administration of CeA fresh leaf juice in neonatal rats (during growth spurt period) enhances their memory, which is probably due to the structural, neurochemical and neurophysiological changes in the brains of these rats.

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