

Neuroprotective effect of *Centella asiatica* leaves extract on substantia nigra neurons - a quantitative study in mice

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Abstract

Centella asiatica is a small medicinal herb, growing in damp soil. In Ayurvedic medicine, *Centella asiatica* is used extensively as brain tonic to enhance learning and memory power. In the present study, the neuroprotective role of *Centella asiatica* (CeA) leaf extract on substantia nigra neurons against stress induced neurodegeneration in mice was investigated. Mice were divided into four groups and treated as follows: (i) Normal control (NC) - remained undisturbed in the cage, (ii) Saline control (SC) - treated with saline, (iii) Stressed group (S) - remained in the restrainer for 6 hours/day for 6 weeks, (iv) Stress + *Centella asiatica* treated group (S+CeA) - stressed and received orally CeA leaf extract throughout the stress period (n=6 in all groups). After 6 weeks, brain was removed and processed for Golgi staining. Substantia nigra neurons were traced using camera lucida focused at 400X magnification. The concentric circle method of Sholl was used to quantify the dendrites. The results showed less number of dendritic branching points and dendritic intersections in stressed group. On the other hand, the number of dendritic branching points and dendritic intersections of substantia nigra neurons in group (iv) were significantly more. The results conclude that, oral intubation of *Centella asiatica* leaves extract increased dendritic branching points and dendritic intersections of substantia nigra neurons.

Keywords: Restraint stress, Substantia nigra, Dendritic branching points, Dendritic intersections

Introduction

The substantia nigra, part of the midbrain which contains neurons that produce the neurotransmitter, dopamine. It appears black in color, hence the name "substantia nigra". It is important for motor planning and processing. Parkinson's disease is a disorder that involves progressive death of neurons in the substantia nigra, leading to motor and cognitive symptoms^[1]. Disturbances in the dopamine neuron activity also lead to another clinical condition - schizophrenia^[2].

Various experimental studies conducted on mice and rats have shown the neuroprotective effect of certain plant extracts like *Gynostemma pentaphyllum*, *Acanthopanax senticosus*, *Juniperus communis*, *Centella asiatica* on substantia nigra neurons^[3,4,5,6].

The anti-stress effect of *Centella asiatica* on the dendritic branching points and intersections of the substantia nigra neurons has not been reported. The effect of restraint stress on the substantia nigra neurons has been reported leading to dendritic atrophy^[7]. The present study was aimed to determine the anti-stress effect of *Centella asiatica* leaf extract on the dendritic branching points and intersections of substantia nigra neurons.

Materials and Methods

Three months old albino mice weighing 30-36 grams were reared and maintained in the central animal house for the study purpose. The mice were kept in a

well ventilated room with 12hrs light and 12hrs dark cycles. Each cage had 4-6 mice with paddy husk as bedding material. There was continuous access to food and water for the mice throughout the day except during stress period of the experiment.

Extraction procedure: Fresh *Centella asiatica* leaves were collected, cleaned and sunshade dried. It was then powdered. Dry powder was weighed and mixed with distilled water at 1:10 ratio and boiled over a low flame for 30 minutes, cooled and decanted. The above procedure was repeated twice. Clear supernatant obtained each time was decanted and then centrifuged (300 rpm for 5 minutes). And 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 leaves extract was done and stored in air tight bottle. For each mouse 500 mg/kg body weight of CeA leaves extract was administered orally throughout the experimental period (6 weeks) in separate groups. Plant extract was dissolved in saline to get the appropriate dilution. Drug was administered orally just before the stress exposure on each day.

Oral intubation: 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 and the drug was delivered slowly.

Restrainer and stress procedure: Wire mesh restrainer was made locally consisting of 12 compartments, was used for restraint stress. The dimensions of each compartment - 2" (length) 1.5"

(breadth) 1.4" (height). Mice were stressed individually by placing within the restrainer for 6hrs/day for 6 weeks. Stress induction and its severeness were assessed by weighing the suprarenal gland at the time of sacrifice and comparing with the normal.

Experimental design:

1. Normal control group (NC) - they remain undisturbed in their home cage.
2. Saline control (SC) – mice in this group received equivolume of normal saline during the experimental period (6 weeks).
3. Stress group (S) – mice in this group were stressed in a wire mesh restrainer 6hr/day for 6 weeks.
4. Stress+ Centella asiatica leaves extract group (S+CeA) - mice in this group were stressed in the same way as in the group-iii, and treated with 500mg/kg/day of aqueous leaves extract of CeA throughout the stress period (i.e. 6 weeks). Drug 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 anesthesia. Brain was removed and processed for rapid Golgi staining (n=8 in each group). Number of dendritic branching points and dendritic intersections were quantified.

Selection of neurons for quantification: 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 400 X magnification. The concentric circle method of Sholl was used to quantify the dendrites. Concentric circles were drawn at 20µm 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 neurons following analysis were done.

1. **Dendritic branching points:** It is a measure of nature of dendritic arborization. The number of dendritic branching points within each concentric circle (between adjacent concentric circles) was counted. In addition, total number of dendritic branching points in each neuron was also counted.
2. **Dendritic intersections:** It is a measure of total length of dendrites. It is a point where a dendrite touches or crosses concentric circle of the Sholl's grid placed over a traced neuron.

Statistical analysis of data: Data obtained from the above experiments were correlated and analysed using one way analysis of variance (ANOVA) followed by Bonferroni's post-test. Student's t-test was applied where ever applicable using statistical software package (Graph pad in Stat).

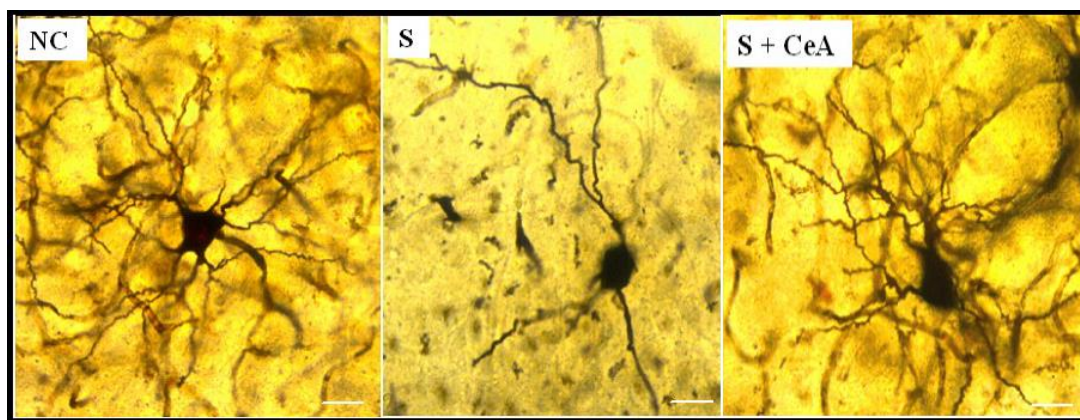


Fig. 1: Photomicrographs of substantia nigra neurons (Golgi staining) in different groups

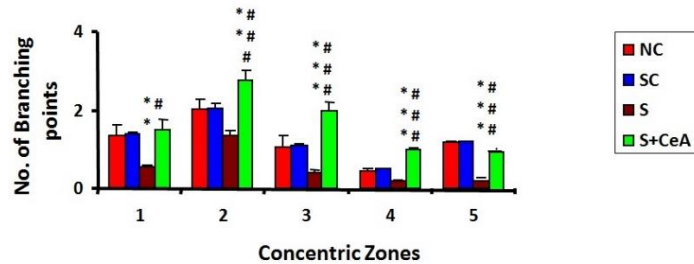
Fig. 1: Shows the Golgi impregnated substantia nigral neurons in Normal control, stressed, Centella asiatica leaves extract treated groups. Note the significantly decreased dendritic arborization in the stressed group (S) compared to normal control (NC). The dendritic arborization in the stressed and CeA treated (S+CeA) group was comparable to control group. Since dendritic morphology of saline group was similar to control group, photograph is not shown. Scale bar = 10µm.

Results

- a. **Dendritic branching points at different concentric zones:** Dendritic branching points were decreased significantly in the stressed group at all concentric zones compared to normal control (P<0.01-0.001). In S + CeA group, dendritic branching points at all concentric zones were significantly increased compared to stressed group (P<0.05 - 0.001, One way ANOVA, Bonferroni's test)

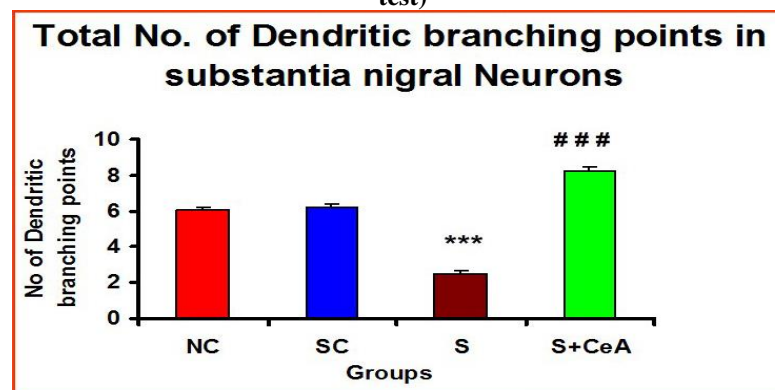
Graph 1: Note there is a significant decrease in dendritic branching points in stressed group (S) compared to control (NC)/ Saline (SC) groups in all concentric zones. It was increased in *Centella asiatica* leaves extract treated groups (S+CeA) in all concentric zones. NC vs S- $**P<0.01$, $***P<0.001$; S vs CeA - $###P<0.001$ (One way ANOVA, Bonferroni's Test)

Dendritic branching points of Substantia nigra neurons



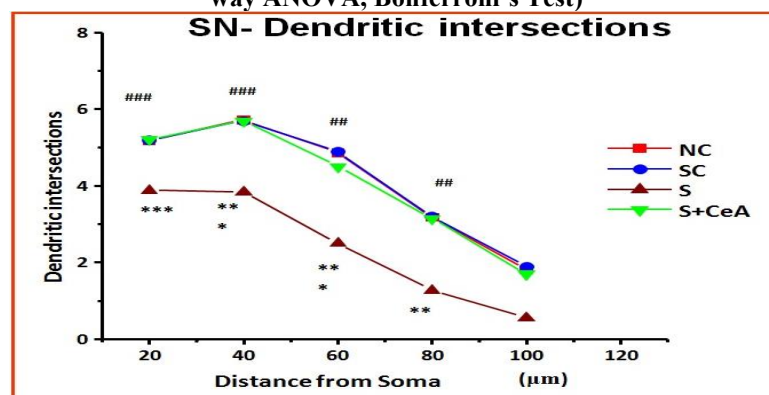
b. **Total number of dendritic branching points:** Total number of dendritic branching points were decreased significantly in the stressed group compared to normal control (6.07 ± 0.12 in control Vs. 2.54 ± 0.17 in stressed, $P<0.001$). In Stressed and treated with *Centella asiatica* leaves extract dendritic branching points were significantly increased compared to stressed group (2.54 ± 0.17 in stressed Vs 8.25 ± 0.25 in S+CeA treated $P<0.001$, One way ANOVA, Bonferroni's test).

Graph 2: Note there is significant decrease in dendritic branching points in stressed group and was increased in S+CeA treated group. NC vs S $***P<0.001$; S vs S+CeA - $###P<0.0001$ (one way ANOVA, Bonferroni's test)



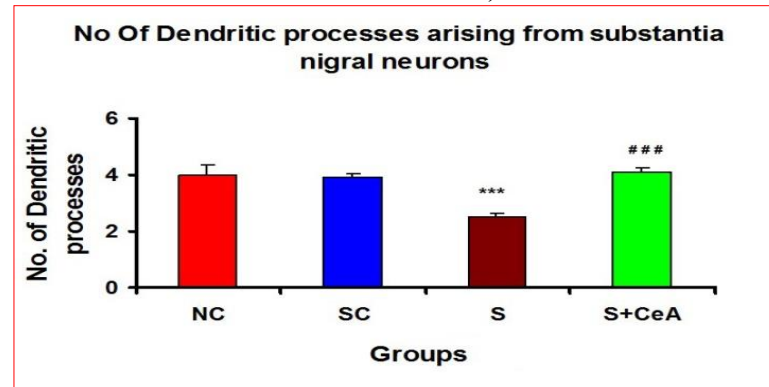
c. **Dendritic intersections:** Dendritic intersections were decreased at $20\mu\text{m}$, $40\mu\text{m}$, $60\mu\text{m}$ and $80\mu\text{m}$ distance from soma in stressed mice compared to normal control and saline control group of mice ($P<0.01 - 0.001$). The dendritic intersections were increased significantly at the same radial distance in S+CeA treated group of mice ($P<0.01 - 0.001$).

Graph 3: Note there is a decrease in dendritic intersections in stressed group, which was increased in *Centella asiatica* leaves extract treated group. NC vs S- $**P<0.01$, $***P<0.001$; S vs CeA- $##P<0.01$, $###P<0.001$ (One way ANOVA, Bonferroni's Test)



- d. **Dendritic processes:** There was a significant reduction in the number of dendritic processes arising from the soma in the substantia nigra pars compacta neurons in the stressed group compared to the normal control and saline control groups (3.98 ± 0.35 in control Vs. 2.5 ± 0.15 in stressed, $P < 0.001$). However when stressed group was compared with S+CeA treated group, the latter had significantly more number of dendritic processes (2.5 ± 0.17 in stressed Vs 4.10 ± 0.15 in S+CeA treated, $P < 0.001$)

Graph 4: Note there is significant decrease in number of dendritic processes in stressed group and was increased in S+CeA treated group. NC vs S * $P < 0.001$; S vs S+CeA - ### $P < 0.001$ (one way ANOVA, Bonferroni's test)**



Discussion

In the present study, we have observed atrophy in the dendritic arborization of substantia nigra neurons which may affect the various functions of substantia nigra. The dendritic atrophy of the substantia nigra neurons may be due to:

Excitotoxicity: The excitotoxicity due to increased release of excitatory neurotransmitter, glutamate may be responsible for the dendritic atrophy of substantia nigra neurons^[8]. Glutamate act on NMDA receptors in substantia nigra to release excess dopamine produced due to exposure to stress^[9]. Release of dopamine either spontaneously or through stimulation is influenced by the glutamate. Interaction between the dopamine and glutamate systems has been involved in many clinical conditions like Parkinson's disease, schizophrenia, stress^[10]. Decrease dopamine level in substantia nigra has been a major cause for Parkinson's disease and increased level of dopamine has been reported to be major factor for schizophrenia^[11]. Glutamate is not the only neurotransmitter involved in dendritic atrophy, other neurotransmitters like noradrenaline and serotonin, are also involved in neuronal damage^[12,13].

Glucocorticoid toxicity: Stress enhances the activity of the adrenocortical axis which in turn elevates circulating glucocorticoid concentrations^[14]. Both stress and glucocorticoids increase glutamate concentrations in the straitum^[15]. Glutamate stimulates the production of brain-derived neurotrophic factor (BDNF), which, in turn, alters glutamate sensitivity of neurons, calcium equilibrium and shape of the neurons. Excessive activation of glutamate receptors, under conditions like oxidative and metabolic stress, may contribute to neuronal dysfunction and degeneration in diseases^[16].

Effects of Centella asiatica leaf extract on neurons:

Centella asiatica (CeA) induce changes in the dendritic morphology of substantia nigra. It protected the neurons from death and reduced the dendritic atrophy in stress condition.

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

Neuroprotectors and antioxidants: The derivatives of asiaticoside, present in the *centella asiatica* extract are effective in protecting the neurons from oxidative damage caused by exposure to excess glutamate^[17]. Accordingly, in the present study the neuroprotective and antioxidant property of *centella asiatica* may be responsible for the neuroprotection against cell death and the deleterious effects of stress and hence increased dendritic arborization.

Conclusion

From the results of our study we conclude that, oral intubation of Centella asiatica plant extract in stressed albino mice leads to the following:

1. CeA leaves extract significantly increase the dendritic branching points and total number of dendritic branching points of substantia nigra neurons in stressed and *centella asiatica* extract treated mice.
2. The extract also increased the dendritic intersections and the number of processes arising from the substantia nigra neurons in stressed and *centella asiatica* extract treated mice.

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