Original Article

Enhancement of Hippocampal CA3 Neuronal Dendritic Arborization by Glycyrrhiza glabra root extract Treatment in Wistar Albino Rats

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Abstract

Background: In the traditional system of medicine, the roots and rhizomes of *Glycyrrhiza glabra* (Gg) (family: *Leguminosae*) have been in clinical use for centuries. **Aim:** In the present study, we investigated the role of aqueous extract of root of Gg treatment on the dendritic morphology of hippocampal Cornu Ammonis area three (CA3) neurons, one of the regions concerned with learning and memory, in 1- month- old male Wistar albino rats. **Materials and Methods:** The aqueous extract of root of Gg was administered orally in four doses (75, 150, 225 and 300 mg/kg) for 4 weeks. After the treatment period, all experimental animals were subjected to spatial learning (Morris water maze, Hebb-William's maze and elevated plus maze) tests. At the end of the spatial memory tests, the rats were deeply anesthetized with Pentobarbitone and killed their brains were removed rapidly and fixed in rapid Golgi fixative. Hippocampal CA3 neurons were traced using camera lucida, and dendritic arborization and intersections were quantified. These data were compared to those of age-matched control rats. **Results:** The aqueous root extract of Gg in the dose of 150 and 225 mg/kg/p.o showed a significant ($P < 0.01$) enhancement of dendritic arborization (dendritic branching points) and dendritic intersections along the length of both apical and basal dendrites in hippocampal (CA3) pyramidal neurons is comparable to control. **Conclusion:** Based on our results obtained, we conclude that constituents present in aqueous root extract of Gg have neuronal dendritic growth stimulating properties.

Key words: Camera lucida, dendritic arborization, glycyrrhiza glabra, hippocampal CA3 neurons

INTRODUCTION

The hippocampus is a major component of the brain of humans and other mammals located bilaterally in the medial temporal lobe, underneath the cortical surface. It belongs to the limbic system and plays important roles in long-term memory and spatial navigation particularly the CA3 subregion of the hippocampus.[1-3]

Traditional herbal extracts have been used to enhancing learning and memory.[4-7] Our earlier studies have shown that Glycyrrhiza glabra (Gg) aqueous root extract treatment (2, 4 and 6 weeks duration of the treatment) in different age grouped (1 and 3 months old) Wistar albino rats enhances both spatial learning ability and retention of learned tasks[8-10] accordingly, the present study was designed to study the effects of Gg root extract on rat hippocampal neurons particularly the CA3 subregion of the hippocampus.

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MATERIALS AND METHODS

Plant material

The roots of Gg were purchased from a local ayuredic store in Udupi, Karnataka, India during 2/4/2012. The material was authentified by the Dr. Krishna Kumar, Chairman, Department of applied Botany, Mangalore University.

Preparation of aqueous root extract

The crude aqueous extract of Gg was prepared by macerating dried powdered root with respective solvent for

24 h. The macerated powdered roots were then extracted by using soxhlet extractor for 36 h, 1-2 cycles/h. The extract was dried and weighed. A brownish black waxy residue with 16% yield was obtained. This aqueous extract of Gg was administrated orally to separate groups of 1-month old male Wistar albino rats in four different doses 75, 150, 225 and 300 mg/kg respectively.

Animals

The experimental protocol was approved during September 2011 by the Institutional Animals Ethics Committee, Yenepoya University and care of laboratory animals was taken as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Rats were housed individually (Animal house, Yenepoya University, Reg.no 347/CPCSEA) in polypropylene cages of standard dimensions (22.5 cm × 35.5 cm × 15 cm) and maintained at temperature (25° C ± 2° C) and light (light period, 08.00-20.00) in a controlled room with relative humidity of 50-55%. Food and water were provided ad libitum. Experiments were carried out between 09:00h and 14:00 h.

Experimental design

Rats were randomly divided into eight groups.

- Group I-: Control ($n = 6$): A known volume of distilled water was administrated orally each day for 4 weeks
- Group II- : Diazepam control ($n = 6$): Diazepam 7 mg/kg was injected i.p. 20 min before the test session
- Group III ($n = 6$): Received 75 mg/kg aqueous extract of Gg orally every day for 4 weeks
- Group IV ($n = 6$): Received 150 mg/kg aqueous extract of Gg orally every day for 4 weeks
- Group V ($n = 6$): Received 225 mg/kg aqueous extract of Gg orally every day for 4 weeks
- Group VI ($n = 6$): Received 300 mg/kg aqueous extract of Gg orally every day for 4 weeks
- Group VII-: Gg 150 mg + Diazepam ($n = 6$): Received 150 mg/kg aqueous extract of Gg orally every day for 4 weeks. Diazepam 7 mg/kg was injected i.p. 20 min before the test session.
  n = number of animals.
- Group VIII-: Gg 225 mg + Diazepam ($n = 6$):Received 225 mg/kg aqueous extract of Gg orally every day for 4 weeks. Diazepam 7 mg/kg was injected i.p. 20 min before the test session.

Rapid Golgi staining procedure

After the treatment period, all experimental animals were subjected to spatial learning (Morris water maze, and elevated plus maze) tests. At the end of the spatial memory tests, the rats were deeply anesthetized with Pentobarbitone and killed; their brains were removed rapidly and fixed in rapid Golgi fixative. Tissues were processed for rapid Golgi staining.

Briefly, tissues were fixed for 5 days in Golgi fixative and impregnated with a 1.5% aqueous silver nitrate solution for 48 h. Sledge microtome sections of 120-μm thickness were excised, dehydrated, cleared and mounted with Distrin plasticizer xylene mounting media.[11]

Camera lucida tracing

From each rat, hippocampal CA3 pyramidal neurons were traced using camera lucida and their dendritic branching points (a measure of dendritic arborization) and dendritic intersections (a measure dendritic length) were quantified.

Quantification of dendritic branching points and dendritic intersections

The concentric circle method of Sholl[12] was used for the dendritic quantification. Concentric circles with a distance of 20 μm between 2 adjacent concentric circles were drawn on a transparent sheet for quantification of dendritic branching points and dendritic intersections.

The number of branching points between the two concentric circles that is each successive 20 μm concentric zone (circle) was counted. The dendritic intersections point (a dendrite intersected a given concentric circle) at each concentric circle were counted. Both branching points and intersections were counted up to a radial distance of 140 μm from the center of the cell body of the CA3 neuron. Mean number of apical and basal dendritic quantification (dendritic branching points and dendritic intersections) in each concentric zone were calculated.

Statistical analysis

Data were analyzed using the ANOVA followed by Dunnett's multiple comparison test. $P < 0.05$ were considered as statistically significant.

RESULTS

Figures 1-4 illustrates Camera lucida tracings (A1, B1, C1, D1, E1, F1, G1 and H1) of Golgi-stained (silver nitrate impregnated) hippocampal CA3 pyramidal neurons (A, B, C, D, E, F, G and H) of control and different doses of the aqueous root extract of Gg treated rats for 4-weeks.

Dendritic quantification of hippocampal CA3 pyramidal neurons

The aqueous root extract of Gg in the dose of 150 and 225 mg/kg/p.o showed significantly ($P < 0.01$) increased numbers of dendritic branching points and dendritic length along the length of both apical and basal dendrites in all

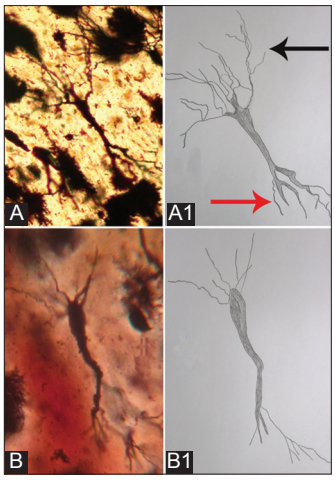

Figure 1: Representative photomicrographs (A-B) and camera lucida tracings (A1-B1) of Golgi-stained hippocampal CA3 neurons. A and A1-Control (Group I); B and B1- Diazepam control (Group II); Black arrow- Basal dendrites of hippocampal CA3 neurons; Red arrow- Apical dendrites of hippocampal CA3 neurons

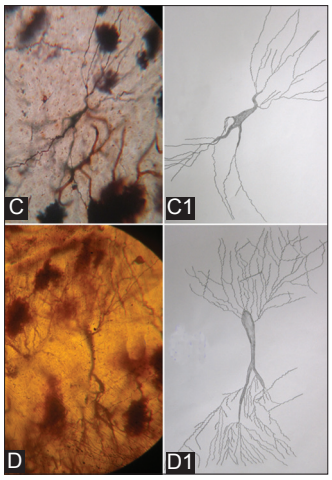

Figure 2: Representative photomicrographs (C-D) and camera lucida tracings (C1-D1) of Golgi-stained hippocampal CA3 neurons. C and C1- hippocampal CA3 neurons of rats treated with 75 mg/kg aqueous extract of Gg orally every day for 4 weeks (Group III); D and D1- hippocampal CA3 neurons of rats treated with 150 mg/kg aqueous extract of Gg orally every day for 4 weeks (Group IV)

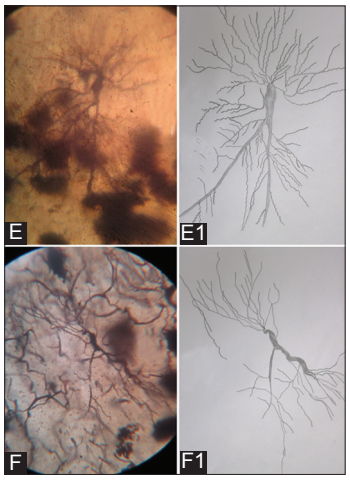

Figure 3: Representative photomicrographs (E-F) and camera lucida tracings (E1- F1) of Golgi-stained hippocampal CA3 neurons. E and E1- hippocampal CA3 neurons of rats treated with 225 mg/kg aqueous extract of Gg orally every day for 4 weeks (Group V); F and F1- hippocampal CA3 neurons of rats treated with 300 mg/kg aqueous extract of Gg orally every day for 4 weeks (Group VI)

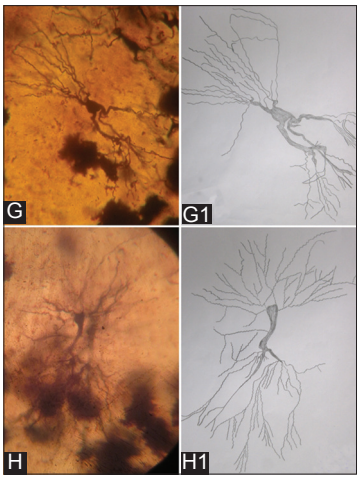

Figure 4: Representative photomicrographs (G-H) and camera lucida tracings (G1-H1) of Golgi-stained hippocampal CA3 neurons of rats treated with aqueous extract of Glycyrrhiza glabra (Gg) for 4 weeks. G and G1- hippocampal CA3 neurons of rats treated with Gg150 mg/kg/p.o + Diazepam 7 mg/kg/i.p (Group VII); H and H1- hippocampal CA3 neurons of rats treated with Gg225 mg/kg/p.o + Diazepam 7 mg/kg/i.p (Group VIII)

the (0-20, 20-40, 40-60, 60-80, 80-100, 100-120, 120-140 μm) concentric zones is comparable to control rats [Tables 1-4].

Furthermore Diazepam induced amnesia reversed by the aqueous root extract of Gg (150 and 225 mg/kg, p.o) has shown a significant ($P < 0.01$) increased numbers of both apical and basal dendritic branching points and dendritic intersections in all the (0-20, 20-40, 40-60, 60-80, 80-100, 100-120, 120-140 μm) concentric zones.

In addition, the aqueous root extract of Gg in the dose of 300 mg/kg/p.o has shown a significant ($P < 0.05$) increased basal dendritic arborization in the 0-20, 20-40, 40-60, 60-80, 80-100, 100-120 μm concentric zones and increased basal dendritic intersections ($P < 0.01$) in the 60-80, 80-100, 100-120, 120-140 μm concentric zones. This dose also shown a significant ($P < 0.01$) increased apical dendritic arborization in the 20-40, 40-60, 60-80, 80-100 μm concentric zones and increased ($P < 0.01$) apical dendritic intersections in the 80-100 and 100-120 μm concentric zones.

DISCUSSION

The dendrites of hippocampal CA3 pyramidal neurons receive inputs from entorhinal cortex, septal area, mamillary

Table 1: Basal dendritic branching points of hippocampal CA3 neurons at different concentric zones in 1-month old male Wistar albino rats (four weeks duration)

Groups	Distance from soma (µm)						
	0-20	20-40	40-60	60-80	80-100	100-120	120-140
Control	0.67±0.21	4.17±0.30	2.55±0.22	2.67±0.21	0.50±0.22	0.00±0.00	0.00±0.00
Diazepam7 mg/kg/i.p	0.00±0.00	0.07±0.21	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Gg75 mg/kg/p.o	1.67±0.21*	3.5±0.42	4.50±0.22	3.33±0.21	0.67±0.21	0.33±0.21	0.00±0.00
Gg150 mg/kg/p.o	3.67±0.33**	9.66±0.55**	8.67±0.67**	8.17±0.95**	11.17±0.48**	9.67±0.33**	4.33±0.21**
Gg225 mg/kg/p.o	6.50±0.22**	18.00±0.36**	12.83±0.48**	8.33±0.95**	8.17±0.54**	6.83±0.48**	4.83±0.16**
Gg300 mg/kg/p.o	1.67±0.21*	6.00±0.36*	4.83±0.30*	5.50±0.22*	2.33±0.21*	2.00±0.63*	0.50±0.22
Gg150 mg/kg/p.o+Diazepam7 mg/kg/i.p	3.16±0.17**	8.50±0.42**	8.33±1.26**	8.33±0.49**	7.17±0.60**	5.33±0.92**	2.67±0.21**
Gg225 mg/kg/p.o+Diazepam7 mg/kg/i.p	3.50±0.22**	9.67±0.67**	16.17±0.30**	10.83±1.17**	6.67±0.42**	4.83±0.79**	2.67±0.21**

n=6; values (number of apical dendritic branching points) are expressed as Mean±SEM;* *P*<0.05,** *P*<0.01 (ANOVA followed by Dunnett's multiple comparison test);
Gg: *Glycyrrhiza glabra*

Table 2: Basal dendritic intersections of hippocampal CA3 neurons at different concentric zones in 1- month old male Wistar albino rats (four weeks duration)

Groups	Distance from soma (µm)						
	0-20	20-40	40-60	60-80	80-100	100-120	120-140
Control	0.17±0.17	1.00±0.25	2.00±0.36	4.33±0.33	3.17±0.17	0.00±0.00	0.00±0.00
Diazepam7 mg/kg/i.p	0.00±0.00	0.50±0.22	0.83±0.40	0.16±0.16	0.00±0.00	0.00±0.00	0.00±0.00
Gg75 mg/kg/p.o	0.33±0.21	0.67±0.21	2.16±0.30	3.67±0.21	3.83±0.17	6.50±0.22**	0.00±0.00
Gg150 mg/kg/p.o	2.33±0.21**	3.00±0.36**	5.33±0.49**	8.17±0.30**	11.83±0.30**	10.5±0.22**	9.83±0.31**
Gg225 mg/kg/p.o	2.67±0.21**	10.83±0.40**	12.67±0.33**	10.83±0.40**	16.50±0.22**	6.50±0.22**	4.33±0.21**
Gg300 mg/kg/p.o	0.33±0.21	0.83±0.16	2.33±0.21	7.00±0.25**	6.00±0.44**	5.67±0.21**	1.33±0.21**
Gg150 mg/kg/p.o+Diazepam7 mg/kg/i.p	2.50±0.22**	3.50±0.22**	4.66±0.21**	8.00±0.36**	7.17±0.54**	10.50±0.34**	6.50±0.22**
Gg225 mg/kg/p.o+Diazepam7 mg/kg/i.p	2.50±0.22**	4.67±0.33**	16.17±0.33**	14.83±0.30**	10.83±0.40**	6.00±0.25**	2.00±0.26**

n=6; values (number of basal dendritic intersections) are expressed as Mean±SEM; * *P*<0.05,** *P*<0.01 (ANOVA followed by Dunnett's multiple comparison test);
Gg: *Glycyrrhiza glabra*

Table 3: Apical dendritic branching points of hippocampal CA3 neurons at different concentric zones in 1-month old male Wistar albino rats (four weeks duration)

Groups	Distance from soma (µm)						
	0-20	20-40	40-60	60-80	80-100	100-120	120-140
Control	0.33±0.21	0.17±0.17	2.50±0.22	1.50±0.22	3.50±0.22	0.00±0.00	0.00±0.00
Diazepam7 mg/kg/i.p	0.00±0.00	0.00±0.00	1.00±0.36	1.00±0.36	1.33±0.21	0.00±0.00	0.00±0.00
Gg75 mg/kg/p.o	1.33±0.21**	2.67±0.42**	2.67±0.21	2.16±0.30	3.16±0.16	0.17±0.17	0.00±0.00
Gg150 mg/kg/p.o	2.50±0.22**	3.33±0.21**	4.17±0.17**	15.33±0.21**	11.67±0.33**	15.50±0.22**	8.33±0.21**
Gg225 mg/kg/p.o	2.50±0.22**	10.17±0.31**	18.83±0.30**	5.67±0.33**	7.83±0.48**	6.50±0.67**	3.33±0.21**
Gg300 mg/kg/p.o	1.00±0.00	2.17±0.17**	10.67±0.49**	3.33±0.21**	5.17±0.30**	0.83±0.30	0.00±0.00
Gg150 mg/kg/p.o+Diazepam7 mg/kg/i.p	2.17±0.17**	2.67±0.21**	4.50±0.22**	4.50±0.22**	7.83±0.40**	9.33±0.21**	3.50±0.22**
Gg225 mg/kg/p.o+Diazepam7 mg/kg/i.p	2.50±0.22**	13.17±0.31**	8.83±0.30**	10.83±0.40**	11.00±0.25**	10.33±0.21**	5.33±0.21**

n=6; values (number of apical dendritic branching points) are expressed as Mean±SEM;* *P*<0.05,** *P*<0.01 (ANOVA followed by Dunnett's multiple comparison test);
Gg: *Glycyrrhiza glabra*

body, dentate granule cells and the contralateral CA3 regions and play an important role in the encoding of new spatial information within short-term memory with duration of seconds and minutes.

It is believed that some areas of the brain particularly the hippocampus vulnerable to glutamate, ischemia, inflammatory processes, repeated psychosocial or oxidative stress,[13-14] may leads to dendritic atrophy in CA3 pyramidal neurons of the hippocampus,[15] accompanied by specific cognitive deficits in spatial learning and memory. Alzheimer's disease and schizophrenia are progressive neurodegenerative disorders associated with loss of neurons in distinct brain areas particularly the hippocampus. Such areas of brain structures has been shown to significantly increase the density of spines and dendritic complexity due to repeated exposure to enriched environments.[16] Increase in the dendritic arborization and dendritic intersections in hippocampal CA3 pyramidal neurons may result in alterations in

Table 4: Apical dendritic intersections of hippocampal CA3 neurons at different concentric zones in1- month old male Wistar albino rats (four weeks duration)

Groups	Distance from soma(μm)						
	0-20	20-40	40-60	60-80	80-100	100-120	120-140
Control	0.00±0.00	0.33±0.21	0.33±0.21	1.83±0.22	3.16±0.30	0.00±0.00	0.00±0.00
Diazepam7 mg/kg/i.p	0.00±0.00	0.00±0.00	1.00±0.36	1.00±0.36	1.33±0.21	0.00±0.00	0.00±0.00
Gg75 mg/kg/p.o	0.00±0.00	1.00±0.25	0.67±0.33	1.50±0.16	4.00±0.25	5.00±0.25**	0.00±0.00
Gg150 mg/kg/p.o	2.67±0.21**	2.50±0.34**	2.33±0.21**	9.33±0.21**	10.17±0.33**	14.00±0.22**	23.33±0.21**
Gg225 mg/kg/p.o	5.50±0.22**	3.00±0.25**	13.33±0.21**	12.33±0.49**	7.50±0.43**	11.00±0.36**	7.16±1.24**
Gg300 mg/kg/p.o	0.00±0.00	0.67±0.33	0.67±0.33	1.17±0.48	10.17±0.17**	7.33±0.21**	0.83±0.30
Gg150 mg/kg/p.o+Diazepam7 mg/kg/i.p	2.50±0.22**	2.00±0.25**	3.17±0.17**	4.33±0.21**	11.67±0.33**	12.33±0.21**	6.67±0.33**
Gg225 mg/kg/p.o+Diazepam7 mg/kg/i.p	5.83±0.30**	6.33±0.21**	6.00±0.36**	6.00±0.36**	7.83±0.48**	13.67±0.21**	14.00±0.37**

n=6; values (number of apical dendritic intersections) are expressed as Mean±SEM; * *P*<0.05,** *P*<0.01 (ANOVA followed by Dunnett's multiple comparison test); Gg: *Glycyrrhiza glabra*.

synaptic connectivity. It may result in alteration in learning and memory

The present study showed that the aqueous root extract of Gg in the dose of 150 and 225 mg/kg/p.o significant ($P < 0.01$) enhancement of dendritic arborization and dendritic intersections in hippocampal CA3 pyramidal neurons. Increase in the dendritic arborization and dendritic intersections in hippocampal CA3 pyramidal neurons may result in alterations in synaptic connectivity, which probably is one reason for the enhanced learning and memory in same rats has been reported previously.[17] Thus the aqueous root extract of Gg may stimulate the release of neuromodulators or neuronal dendritic growth stimulating factors that alter the activity of neurotransmitters that are involved in learning and memory, which thereby contributes to enhanced learning and memory.

CONCLUSION

In conclusion, the aqueous root extract of Gg in the dose of 150 and 225 mg/kg/p.o showed a significant ($P < 0.01$) enhancement of dendritic arborization and dendritic intersections in hippocampal CA3 pyramidal neurons is comparable to the control. Based on our results obtained, we conclude that constituents present in aqueous extract of root of Gg have neuronal dendritic growth stimulating properties.

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