Neurotrophic Factors Mediate Memory Enhancing Property of Ethanolic Extract of *Liriope platyphylla* in Mice

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Abstract – The roots of *Liriope platyphylla* (Liliaceae) are widely used in traditional Chinese medicine. In the present study, we investigated the effects of ethanol (70%) extract of the roots of *Liriope platyphylla* (ELP70) on learning and memory using behavioral and immunohistochemical methods in mice. Control animals were treated with vehicle (10% Tween 80). With sub-chronic treatments of ELP70 (p.o.) for 14 days, the latency time was significantly increased compared with that of the vehicle-treated control group (50, 100 and 200 mg/kg; $P < 0.05$). Moreover, immunopositive cells for brain derived neurotrophic factor (BDNF) were significantly increased in the hippocampal dentate gyrus and CA1 regions after ELP70 treatments for 14 days (50, 100 and 200 mg/kg; $P < 0.05$). In addition, those cells for nerve growth factor (NGF) were also increased in the hippocampal dentate gyrus region (50, 100 and 200 mg/kg; $P < 0.05$). These results suggest that the sub-chronic administration of ELP70 improves learning and memory, and that their beneficial effects are mediated, in part, by the enhancement of BDNF or NGF expression.

Keywords □ *Liriope platyphylla*, Memory, Passive avoidance test, NGF, BDNF

INTRODUCTION

Brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are member of the neurotrophin family known to play important roles in the survival, growth, and maintenance of neurons during development (Leibrock et al., 1989; Barde, 1994). Several lines of evidences suggest that BDNF and NGF are essential to modulate synaptic-plasticity in the adult brain. For example, BDNF regulates axonal and dendritic branching and remodeling (Shimada et al., 1998; Lom & Cohen-Cory, 1999; McAllister et al., 1997; Yacoubian & Lo, 2000), synaptogenesis in arborizing axon terminals (Alsina et al., 2001), the

efficacy of synaptic transmission (Lohof et al., 1993; Kang & Schuman, 1995; Boulanger & Poo, 1999; Kafitz et al., 1999), and the functional maturation of excitatory and inhibitory synapses (Seil & Drake-Baumann, 2000; Rutherford et al., 1998; Vicario-Abejon et al., 1998). BDNF gene deletion or inhibition produces a deficit in long-term potentiation (Figurov et al., 1996; Kang et al., 1997). Moreover, NGF promotes the survival and functioning of cholinergic neurons in the basal forebrain (Chen et al., 1997). These neurons project to the hippocampus and are believed to be important for memory processes, which are specifically affected in Alzheimer's disease. Thus, BDNF and NGF play critical roles in the learning and memory process (Nguyen & Kandel, 1996; Chen et al., 1997).

Liriope platyphylla Wang et Tang (Liliaceae) is widely employed for respiratory disease including cough and sputum in traditional Chinese prescriptions. With regards to the phar-

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macological effect of *L. platyphylla*, antibacterial and anticancer effects have been reported (Kim *et al.*, 2002). Moreover, buthanol fraction of *L. platyphylla* enhances the nerve growth factor (NGF) synthesis in the primary astrocyte, and NGF released induces the neurite outgrowth of PC12 cells (Hur *et al.*, 2004). These observations suggest a possibility that *L. platyphylla* affect in memory process through the enhancement of neurotrophic factors. In the present study, we want to confirm this possibility by the treatment with ethanolic extract of *Liriope platyphylla* (ELP70) using behavioral and immunohistochemical methods. We employed the passive avoidance task for learning and memory behavior in mice and investigated the changes of BDNF and NGF expression in the hippocampal formation using immunohistochemical methods.

MATERIALS AND METHODS

Animals

Male ICR mice (25-30 g) were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea). The animals were housed 5 or 6 per cage, allowed access to water and food ad libitum, and maintained under a constant temperature ($23\pm1^{\circ}\text{C}$) and humidity ($60\pm10\%$) under a 12-h light/ dark cycle (light on 07.30 - 19.30 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea.

Materials

Anti-NGF antibody and anti-BDNF antibody were purchased from Abcam (UK), and Santa Cruz Biotech (USA), respectively. The roots of *Liriope platyphylla* were obtained from an herbal supplier in Seoul, Korea, and voucher specimens (KHUOPS-03-19) were deposited at the herbarium of the College of Pharmacy, Kyung Hee University (Seoul, Korea). The material was authenticated by Emeritus Professor Chang Soo Yook of the Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University. All the other materials were of the highest grade commercially available.

Sample preparation

The dried the roots of *Liriope platyphylla* (1 kg) were extracted with 70% EtOH three times under an ultrasonic apparatus. The ethanolic solution obtained was filtered, concen-

trated in a water bath under vacuum, removed solvent in vacuum, frozen and lyophilized (Eyela, model FDU-2000, Japan) to yield the ethanolic extract (yield 12.5%), which was then stored at -20°C until required. ELP70 was freshly suspended in 10 % Tween 80 solution and orally administered for 14 days.

Passive avoidance task

Passive avoidance performance was carried out in two identical light and dark square boxes ($20 \times 20 \times 20$ cm, respectively) separated by a guillotine door (5×5 cm), as described in our previous report (Kim *et al.*, 2006). The illuminated compartment contained a 50 W bulb, and the floor of the non-illuminated compartment was composed of 2 mm stainless steel rods spaced 1 cm apart. Mice were initially placed in the illuminated compartment for the acquisition trial and the door between the two compartments was opened 10 s later. When mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.25 mA) of 3 s durations was delivered through the stainless steel rods. One hour after the final administration of ELP70 (25, 50 100, or 200 mg/kg, p.o.) on the 14th day, the mice were placed in the illuminated compartment for the acquisition trials. Control animals were treated with 10% Tween 80 solution only. Twenty-four hours after acquisition trial, the mice were again placed in the illuminated compartment for the retention trials. The time taken for a mouse to enter the dark compartment after door opening was measured as latency times in both acquisition and retention trials.

Tissue preparation

After the passive avoidance task, mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and perfused transcardially 0.1 M phosphate buffer (pH 7.4) followed by ice-cold 4% paraformaldehyde. Brains were removed and post-fixed in phosphate buffer (0.05 M, pH 7.4) containing 4% paraformaldehyde overnight and then immersed in 30% sucrose solution (in 0.05 M phosphate-buffered saline), and stored at 4°C until sectioning. The frozen brain were coronally sectioned on a cryostat ($30\text{ }\mu\text{m}$) and then stored in storing solution at 4°C .

Immunohistochemistry

Free floating sections were incubated for 24 h in PBS (4°C) containing anti- NGF antibody (1:1000 dilution) or anti-BDNF antibody (1:1000 dilution), 0.3% Triton X-100, 0.5 mg/ml of

bovine serum albumin, and 1.5% normal horse serum. The sections were then incubated for 90 min with biotinylated secondary antibody (1:200 dilution, Vector, Burlingame, CA, USA), and then with avidin-biotin-peroxidase complex (1:100 dilution, Vector, Burlingame, CA, USA) for 1 h at room temperature. Finally, the sections were reacted with 0.02% 3, 3'-diaminobenzidine and 0.01% H₂O₂ for about 3 min. After each incubation step, sections were washed three times with PBS. Sections were mounted on gelatin-coated slides, dehydrated in ascending alcohol concentrations and cleared in xylene.

Quantitation of data and Statistical Analysis

Immunopositive cells were counted with a computerized image analysis system (Leica Microsystems AG Wetzlar, Germany). Cell counts were carried out in the dentate gyrus and CA1 regions in hippocampal area in five mice per group and in a blinded manner, which allowed statistical analysis. Values are expressed as mean $\pm$ S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of ELP70 on the passive avoidance task

We assessed whether ELP70 improves memory function in the passive avoidance task which is largely dependent on long-term memory. When placed on the bright side of a step-through box, mice quickly entered into the dark compartment. When mice were conditioned using a mild foot shock (0.25 mA) after entering the dark compartment, they hesitated to re-enter the dark compartment when tested 24 h later. The sub-chronic treatment of ELP (25, 50, 100, or 200 mg/kg) showed a significant group effect on the step-through latency in retention trial [$F(4, 45) = 3.74, P < 0.05$] (Fig. 1). The step-through latencies were significantly longer by ELP70 treatment for 14 days (50, 100, and 200 mg/kg) compared with those of vehicle-treated control group ($P < 0.05$, Fig. 1). In the acquisition trial, there were no significant differences of step-through latency across all groups.

Effect of ELP70 on the immunoreactivities of BDNF and NGF

With the sub-chronic administration of ELP70, BDNF immunoreactive cells were markedly increased within the hippocampal CA1 and dentate gyrus regions in a dose dependant

Fig. 1. Effect of sub-chronic administration of ethanolic extract of *Liriope platyphylla* (ELP70) in the passive avoidance task. ELP70 was administered orally for 14 days. The last administration of ELP70 (25, 50, 100, and 200 mg/kg, p.o.) or the same volume of 10% Tween 80 solution was accomplished 60 min before the acquisition trials. The retention trials were carried out 24 h after acquisition trials. Data represent means $\pm$ S.E.M. ($n = 10$). * $P < 0.05$, compared with the vehicle control group.

manner ($P < 0.05$, Fig. 2). Especially, BDNF positive cells in dentate gyrus region were higher than those in CA1 region. The NGF immunoreactive cells were also increased by treatment of ELP70 in dentate gyrus region ($P < 0.05$, Fig. 3). However, those cells were not observed in CA1 region (Fig. 3A1-D1).

DISCUSSION

In the present study, it was found that sub-chronic treatment with ELP70 enhanced memory performances in the passive avoidance task in mice. Furthermore, ELP70 also increased the expressions of neurotrophic factors including NGF and BDNF in the hippocampal formation. These results suggest that the memory enhancing properties of ELP70 may be associated with the increase of expressions of neurotrophic factors in the hippocampal formation.

Previously, we observed that there were no significant changes in the latency time in the passive avoidance test with an acute single administration of ELP70 (dose ranges; 25-200 mg/kg, p.o.) (data not shown). Recently, Hur *et al* (2004) reported that butanol fraction of *L. platyphylla* enhanced the NGF synthesis in the primary astrocyte, and NGF released induced the neurite outgrowth of PC12 cells. Therefore, we assumed that prolonged administration of ELP70 enough for inducing neurotrophic factor expressions could affect learning

Fig. 2. Representative photomicrographs of the BDNF positive cells in the hippocampal (A-D), dentate gyrus (A-1, B-1, C-1, and D-1), CA1 regions (A-2, B-2, C-2, and D-2), and the number of BDNF positive cells in the dentate gyrus (DG) and CA1 regions (E) after administration of vehicle (A-A-2) or ethanolic extract of *Liriope platyphylla* (ELP70) at 50 mg/kg (B-B-2), 100 mg/kg (C-C-2), or 200 mg/kg (D-D-2) for 14 days. Data represent means $\pm$ S.E.M ($n=5$). *,# $P<0.05$, compared with the vehicle control group. The bar represents 100 μ m for A-D (magnification: 40 X). The bar represents 50 μ m for others (magnification: 400 X).

and memory. To confirm this hypothesis, the ELP70 was administered for 14 days. In the present study, sub-chronic administration of ELP70 increased the latency time in the passive avoidance task versus vehicle-treated control group in a dose dependant manner. However, during the acquisition trial, no differences in latencies were observed between any groups, demonstrating no significant effects of ELP70 on the general

Fig. 3. Representative photomicrographs of the NGF positive cells in the hippocampal (A-D), dentate gyrus (A-1, B-1, C-1 D-1), CA1 regions (A-2, B-2, C-2, D-2), and the number of NGF positive cells in the dentate gyrus region (E) after administration of vehicle (A-A-2) or ethanolic extract of *Liriope platyphylla* (ELP70) at 50 mg/kg (B-B-2), 100 mg/kg (C-C-2), or 200 mg/kg (D-D-2) ethanolic extract of *Liriope platyphylla* (ELP70) for 14 days. Data represent means $\pm$ S.E.M ($n=5$). * $P<0.05$, compared with the vehicle control group. The bar represents 100 μ m for A-D (magnification: 40 X). The bar represents 50 μ m for others (magnification: 400 X).

behaviors. These behavioral results suggest that sub-chronic treatment of ELP70 might increase the expression of some molecules related with memory performances, such as neurotrophic factors, because single treatment of ELP70 did not show any significant changes in the passive avoidance task.

NGF is the first identified neurotrophic factor and displays a

restricted target population. In the peripheral nervous system, it acts on sympathetic neurons as well as sensory neurons involved in nociception and temperature sensation (Cahill *et al.*, 2003). In the central nervous system, on the other hand, NGF promotes the survival and functioning of cholinergic neurons in the basal forebrain, suggesting that the increase of NGF expression is related with memory performances (Chen *et al.*, 1997; Gutierrez *et al.*, 1997). If ELP70 induces NGF expression *in vivo*, it would be involved in the memory enhancement because butanol fraction of *L. platyphylla* enhances the NGF synthesis in the primary astrocyte (Hur *et al.*, 2004). To confirm this idea, we carried out NGF immunostaining in the hippocampal region. The sub-chronic ELP70 treatment dramatically increased NGF expressions in the hippocampal dentate gyrus region. The immunopositive cells for NGF in the ELP70-treated group were increased to approximately 297% (50 mg/kg), 477% (100 mg/kg), and 481% (200 mg/kg) of the vehicle-treated control group. In the CA1 region, however, there are no changes of NGF immunopositive cells. Although we did not know the reasons why the NGF expression was not changed in the CA1 region, the enhancement of NGF expression is likely involved in the ELP70-induced memory enhancement. This result is also supported by which exogenous NGF reversed the age-dependent changes in cholinergic basal forebrain neurons and improved performance in spatial memory tasks (Bruno *et al.*, 2004).

BDNF has emerged as a major regulator of synaptic transmission and plasticity at adult synapses in many regions of the CNS. This unique role within the neurotrophin family fits with the widespread distribution of BDNF and the co-localization of BDNF and its receptor, TrkB, at glutamate synapses. The versatility of BDNF is emphasized by its contribution to a range of adaptive neuronal responses including long-term potentiation (LTP), long-term depression (LTD), certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability (Desai *et al.*, 1999; Asztely *et al.*, 2000; kegaya *et al.*, 2002; Maffei, 2002). Moreover, BDNF plays a vital role in neuronal survival, growth (Lindsay *et al.*, 1994) and synaptic plasticity (Thoenen, 1995; Schinder and Poo, 2000). Some studies have shown that hippocampal BDNF expression increases during spatial learning (Gooney *et al.*, 2002; Mizuno *et al.*, 2000), whereas BDNF knockout mice show deficits in spatial learning (Linnarsson *et al.*, 1997) and synaptic plasticity (Korte *et al.*, 1995). Moreover, administration of BDNF improves performance in a spatial memory task (Alonso *et al.*, 2002; Cirulli *et al.*, 2004). If sub-chronic ELP70

treatment can affect the BDNF expression in the hippocampal area, BDNF would also be involved in ELP70-related learning and memory performances. ELP70 treatment increased BDNF to approximately 230% (100 mg/kg) and 330% (200 mg/kg) of the vehicle-treated control group in the hippocampal dentate gyrus region. On the contrary to the NGF expression, in the hippocampal CA1 region, BDNF expression also increased to 340% (100 mg/kg) and 323% (200 mg/kg) of the vehicle-treated control group, respectively. Although we did not find whether the blockade of the increase of NGF or BDNF expressions by ELP70 can deteriorate memory performances, it is likely that sub-chronic administration of ELP70 would enhance memory performances through the increase of NGF or BDNF expressions. Until now, however, it is unclear that the same mechanism or same compound is involved in the increase of NGF and BDNF expressions related with the memory performances. We are currently working on the isolation of active compound in ELP70 using an activity-guided fractionation approach.

From the above behavioral and immunohistochemical data, it can be concluded that ELP70 has an ability to improve learning and memory through the increase of NGF and BDNF expressions in the hippocampus.

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