Abstract:

Bacopa monnieri (L.) Wettst. (BM) is a well-known medicinal plant traditionally used in South-east Asian countries as a brain or nerve tonic. This study aimed to clarify the effects of alcoholic extract of BM on cognitive deficits caused in olfactory bulbectomized (OBX) mice and the underlying molecular mechanisms of its action.

Methods: OBX mice were daily treated with BM (50 – 100 mg/kg per day, p.o) or the reference drug tacrine (2.5 mg/kg per day, i.p.) 1 week before and continuously after 3 days of OBX. Cognitive performances of the animals was analyzed by the novel object recognition test, modified Y maze test, and fear conditioning test to elucidate non-spatial short term memory, spatial working memory, and long-term memory, respectively. After completing the behavioral experiments, the animals were decapitated and the brain tissues were dissected for neurochemical and immunohistochemical studies.

Results: In agreement with our previous study, OBX caused impairment of non-spatial short term memory, spatial working memory, and long-term fair memory. BM administration as well as tacrine treatment significantly ameliorated OBX-induced memory disturbances and the effect of BM in the modified Y-maze test was reversed by scopolamine, a muscarinic receptor antagonist. BM and tacrine enhanced phosphorylation of N-methyl-D-aspartate receptor, calmodulin-dependent kinase (CaMKII), and cyclic AMP-responsive element binding protein (CREB), neural signaling proteins implicated in synaptic plasticity-related-neuronal signaling, and BDNF mRNA in the hippocampi of OBX mice. BM administration also reversed the down-regulated expression of hippocampal choline acetyltransferase (ChAT), a cholinergic marker protein, inhibited ex vivo activity of acetylcholinesterase in the cortices, and the decreased number of the septal cholinergic neurons in the OBX animals.

Conclusion: The present study indicated that that BM treatment ameliorated cognition func-
tion of OBX mice via a mechanism implicated in enhancement of synaptic plasticity-related neuronal signaling and BDNF transcription and that protection of cholinergic systems from OBX-induced neuronal damage may in part play an important role in this mechanism.

1. Introduction

Alzheimer’s disease (AD) mainly affects elderly individuals, and, because of the ageing of populations worldwide, this disorder is reaching epidemic proportions, with a large human, social, and economic burden (1). Therefore, new drug discovery and the establishment of new therapeutic treatments for AD are greatly needed.

*Bacopa monnieri* (L.) Wettst. (BM) is a well-known traditional medicinal plant used in India and other south-east Asian countries as a brain or nerve tonic. Lines of evidence show that BM enhances learning and memory in neonatal and adult healthy rats (2). Chatterjee et al (3) demonstrated that BM at the dose of 80 mg/kg (po) exhibits an anxiolytic and anti-depressant activities in mice. Moreover, BM shows neuroprotective effects on epilepsy (4), amnesia (5), and attenuates memory deficits induced by ischemia (6) or intracerebroventricular administration of colchicine (7) in rats. In clinical studies, it has been reported that BM can reduce state of anxiety in healthy adults (8, 9) and improve attention, cognitive processing, and working memory in healthy older persons (10). Accumulated scientific evidence, however, is still needed not only to clarify the mechanism(s) underlying the action of BM but also to promote more possible usages of BM in dementia therapy.

Olfactory bulbectomy (OBX) in rodent has been widely used as a model of cognitive and emotional dysfunction including AD (11-13). Indeed, evidence indicates that the impairment of olfactory perceptual acuity not only is present at the early stage of AD (14) but also observed in mild cognitive disorder (MCI) patients (14) as well as in a transgenic AD model of mice over-expressing a mutant form of the human amyloid-β- precursor protein (15). Moreover, OBX induces elevation of Aβ level in the brain (16) and neurodegeneration of septo-hippocampal cholinergic innervation in rodents (12, 17). In this study, we elucidated the anti-dementia effects and the mechanism underlying the action of BM in an OBX model of mice. Our findings have clearly demonstrated that BM ameliorates cognitive deficits of OBX mice and enhances neuroplasticity-related neuronal signaling and BDNF mRNA expression in part via stimulation/protection of central cholinergic systems

2. Materials and Methods

2.1. Animals

The study was conducted according to the experimental protocols as described in Fig. 1. Male ddY mice (Japan SLC Inc., Shizuoka, Japan) were obtained at the age of 9 weeks old. The animals were habituated to the laboratory animal room for at least 1 week before surgery. Food and water were available ad libitum. Housing was thermostatically maintained at 24 ± 1°C with constant humidity (65%) and a 12-h light-dark cycle (lights on: 07:00 – 19:00). The behavioral
experiments were performed during the light phase from 9:00 to 18:00. The present studies were conducted in accordance with the Guiding Principles (NIH publication #85-23, revised in 1985) for the Care and Use of Animals and were approved by the Institutional Animal Use and Care Committee of the University of Toyama.

2.2. Preparation of Plant extract

*Bacopa monnieri* was collected in Ho Chi Minh city, Vietnam, in 2010. The plant samples were identified by Dr. Pham Thanh Huyen, a botanist of National Institute of Medicinal Materials (Vietnam). The aerial part was cut into small pieces 1-2 cm in length and dried at 60°C in a hot air oven. The herb then was lixiviated in 95% alcohol (1:12 w/v) for 48 h at room temperature. After filtration, the extract was freeze-dried to yield *Bacopa monnieri* alcohol extract (BM, 7.2% of moisture) and kept until use. Yield of the extraction from the dried herb was calculated as 11.9% (w/w).

2.3. Surgical operation

OBX of mice was conducted as previously reported (12, 13). Briefly, the mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and fixed on stereotactic instruments (Narishige, Tokyo, Japan). The skull covering the bulbs was exposed by skin incision, 1% lidocaine solution was used as a local anesthetic and then a 1 mm burr hole was drilled. The bilateral bulbs were aspirated through a syringe and the cavity of the bulbs was filled with hemostatic gelatin sponge. After completing the behavioral studies, all the animals were sacrificed and the operated lesion was verified visually. The data from animals with less than 70% removal or with no intact cortex were excluded from the analysis. Sham operation was performed in a similar way without removal of the bulb. At the end of the experiments, the olfactory bulbs of sham group mice were confirmed to be intact.

2.4. Drug administration

Except in specially stated cases, either vehicle water or test drugs were administered daily from 3 days after surgery during the experimental period. On a behavioral testing day, admin-
istration was conducted 1 h before the testing. Sham group of mice and OBX control group mice were per orally administered water. Reference standard drugs, tacrine (THA; 9-amino-1,2,3,4-tetrahydro-acridine HCl) were dissolved in 0.9% saline and administered once daily at doses of 2.5 mg/kg (i.p.). The BM extract was dissolved in water and given per orally at daily doses of 50 mg/kg.

2.5. Fear conditioning test

The fear conditioning test was conducted according to the previously described method (12, 18) with a slight modification. Briefly, the chamber for fear conditioning consisted of a transparent acrylic chamber (30×30×30 cm) and a stainless-steel grid floor equipped with an electric shock generator/scrambler SGS-002R, CS Controller CSS-001R, and Cycle TimerCMTR (Muro-machi Kikai. Co. Ltd., Tokyo, Japan). The apparatus was placed in a sound-proof observation box (MC-050/CM, Muromachi Kikai, Co. Ltd., Tokyo, Japan) through which the auditory tone (2.9 kHz, 80 dB) (SonalertR, Mallory Sonalert Products Inc., Indianapolis, IN, USA) was delivered to the animal. In the training sessions performed 9 days after the surgery, the animals were placed individually into the fear conditioning chamber and allowed to explore freely for 4 min. Then, they received an acoustic tone (2.9 kHz, 20 s, 80 dB) co-terminated with electric footshocks (0.8 mA, 2 s). The tone-footshock pairing was continuously repeated 5 times with a 1-min interval.

One min after the final footshock delivery, the mice were returned to their home cage. The test sessions were conducted to measure freezing responses to the context and auditory tone 1 day or 5 days after the training, respectively. For the contextual memory test, 1 day after the training, the mice were placed in the same chamber to provide the contextual stimuli and allowed to move freely for 6 min. Behavioral responses recorded for 5 min from 1 min after the animal was placed in the chamber were analyzed to measure freezing behavior as an index of contextual memory. Test phase for auditory-dependent fear memory was conducted 5 day after training trial. The mice were placed in the chamber for 4 min and then received the tone for 2 min. The freezing behavior during the 2-min period was recorded as auditory-dependent fear memory. The animal behavior was video-recorded and analyzed automatically by Smart® system. Freezing was defined as the absence of any movement except for those related to respiration. Footshock-naive mice were used as controls that likely exhibit pseudo-positive freezing response.

2.6. Neurochemical study

2.6.1. Western blotting

Western blotting was performed as previously described (19, 20). We used the following primary antibodies: anti-NMDAR1 rabbit polyclonal antibody (1:1000 dilution), anti-phospho-NMDAR1 (p-NMDAR1) (pSer896) rabbit polyclonal antibody (1:1000 dilution) (Cell Signaling Technology, USA), anti-GluR1 (AMPA subtype) rabbit polyclonal (1:1000 dilution), anti-phosho-GluR1 (pSer 831) rabbit polyclonal antibody (1:1000 dilution) (Sigma–Aldrich, Co., St. Louis, USA), anti-CaMKIIα (A-1: sc-13141) mouse monoclonal antibody (1:1000 dilution), anti-phospho-CaM-
KII (p-CaMKII) (pThr286) rabbit polyclonal antibody (1:1000 dilution), anti-CREB (48H2) rabbit monoclonal antibody (1:1000 dilution), anti-phospho-CREB (p-CREB) (pSer133) rabbit monoclonal antibody (1:1000 dilution), anti-PKCa rabbit polyclonal antibody (1:1000 dilution), anti-phospho-PKCa/βII (pThr638/641) polyclonal antibody (1:1000) (Cell Signaling Technology, USA), anti-choline acetyltransferase goat polyclonal antibody (1:5000 dilution) (AB-144P, Millipore, CA, USA) and anti-b actin mouse monoclonal antibody (1:10,000 dilution, Abcam®, Cambridge, UK).

The immune complexes were detected by the enhanced chemiluminescence method (ImmobilonTM Western Chemiluminescent HRP Substrate) (Millipore, Tumecula, CA, USA) and imaged using Lumino Image Analyzer LAS-4000R (Fuji Film, Tokyo, Japan). The quantity of immune-reactive bands was analyzed using ImageQuant TL software (GE Healthcare, Buckinghamshire, UK). The expression levels of each membrane were re-probed using a Blot Restore Membrane Rejuvenation Kit (Millipore, Tumecula, CA, USA).

2.6.2. Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was conducted as previously described (21, 22). Briefly, the animals were decapitated after completing the behavioral experiments. The hippocampus were dissected out and kept at -80°C until use. Total RNA was extracted from the cortex with Sepazol® (Nacalai Tesque, Japan) according to the manufacturer’s instructions. First-strand cDNA was synthesized with oligo (dT) primers and M-MLV reverse transcriptase® (Invitrogen, USA) and was used as a template for real-time PCR. Quantitative real-time PCR was carried out with Fast SYBR Green Master Mix and the StepOne Real-time PCR System® (Applied BioSystem, USA). The following primer sets of BDNF and b-actin were designed by Perfect Real Time support system (Takara Bio Inc., Japan): BDNF (NM_007540): 5’-AGCTGAGCTGTGTGACAGT-3’ (forward) and 5’-TCCATAGTAAGGGCCCGAAC-3’ (reverse); β-actin (NM_007393): 5’-CATCCGTAAGACCTCTATGCAAC-3’ (forward) and 5’-ATGGAGCCACCGATCC ACA-3’ (reverse). Melting curve analysis of each gene was performed every time after amplification. In all reactions, b-actin mRNA was used as a control to which the results were normalized. Standard curves of the log concentration of each gene vs. cycle threshold were plotted to prove inverse linear correlations. The correlation coefficients for standard curves of target genes were 0.9965 to 0.998.

2.6.3. Ex vivo and in vitro measurements of cholinesterase activity in the brain

Ex vivo measurement: After completing the behavioral experiments, mice were decapitated and the frontal cortices were dissected out and kept at −80°C until use. Determination of cholinesterase activity was performed on the basis of the colorimetric method as previously described (12, 21, 23). Briefly, the frozen cortex was weighed and homogenized in 10-times volume of 0.1 M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000×g at 4°C for 20 min, the clear supernatants were collected and served as the enzyme source. Cholinesterase activity was determined in 10 μl aliquots of homogenates (run as triplicates) in a 96-well flat-bottom microplate. The reaction was started by adding 8 μl of 10 mM 5,5′-dithiobis-2-nitrobenzoic
acid (DTNB), 16 μl of 7.5 mM acetylthiocholine (ATCI) and 201 μl of 0.1 M sodium phosphate buffer (pH 8.0). The spectrophotometric absorption at 405 nm during a 3 min incubation period at 25°C was quantitatively measured using a microplate reader (Sunrise Classic; TECAN Japan, Kawasaki) and is expressed as nmol ACh hydrolyzed/min/mg tissue.

In vitro measurement: The assay for measuring AChE activity was modified from the assay described by Ellman et al. (23) and Ingkaninan et al. (24). Frontal cortex supernatants were obtained from naïve ddY mice as the enzyme source. Cholinesterase activity was determined as described above.

2.7. Data analysis

The data are expressed as the mean ± SEM. The data obtained from the behavioral tests and neurochemical experiments were analyzed by paired and unpaired Student’s t-test or one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison test (Dunnett’s method) as appropriate. Differences of P<0.05 were considered as significant. The analysis was conducted using SigmaStat® ver. 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

3. Results

3.1. BM ameliorates OBX-induced long-term memory deficit in the fear conditioning test

Fear conditioning test was used to clarify the effect of BM treatment on long-term memory deficit in OBX mice. In this test, mice were fear-conditioned to the context and auditory stimuli by electrical footshocks as unconditioned stimuli. Hippocampus- dependent memory and amygdala-dependent memory were analysed as freezing responses of each animal group to the contextual and auditory stimuli after 1 day and 5 days training trial, respectively (Fig 4A). Freezing responses to the context and auditory stimuli of vehicle-treated OBX mice were markedly decreased compared with those of the footshocked sham animals. However, daily treatment of BM and tacrine significantly increased in the freezing time of OBX mice in both contextual and auditory test, indicating the improvement in hippocampus- and amygdala dependent memory formation in OBX mice (Fig 4B, 4C).

3.2. BM facilitates neuroplasticity-related neuro-signaling pathway in the hippocampus of OBX mice

In order to understand the molecular mechanism(s) underlying BM-induced improvement of cognitive deficits in OBX mice, we examined the effects of BM on neuroplasticity-related neuro-signaling pathway in the hippocampus of OBX mice. Western blotting data showed that the total protein expression level of NMDAR1 (NMDAR subunit), GluR1 (AMPA subunit), α-CaMKII or CREB have no change under the effect of OBX or drug treatment (Fig. 5). However, vehicle-treated OBX mice showed a significantly reduced in the levels of pSer896-NMDAR1, pSer831-GluR1 and pThr286-CaMKII compared with sham animals. BM and tacrine treatment reversed the decrease in levels of pSer896-NMDAR1 and pThr286-CaMKII but these treatments
have no influence on the levels of pSer831-GluR1 in OBX mice (Fig. 5 A, B, C, D). No difference in the levels of pSer133-CREB was observed between sham and OBX animals. Interestingly, pSer133-CREB was over-expressed in BM and tacrine-treated OBX mice (Fig. 5 E).

We also measured the expression levels of BDNF gene transcript which is a functional molecule downstream of the transcriptional activity of CREB, via CREB phosphorylation (Figure 6). OBX mice had a significantly reduced level of BDNF mRNA in the hippocampus (P<0.05). However, daily administration of BM and tacrine to OBX mice significantly reversed the decreases in the expression levels of BDNF (P<0.05) (Fig. 6).

Since the activation of PKC is necessary for maintenance of the LTP response, we identified the expression levels of PKCa and pThr638-PKCα/pThr641 PKCβin the hippocampus of each mouse groups. The expression level of PKCa and pThr638-PKCα/pThr641 PKCβ of each animal group were found no significant difference.

### 3.3. The effects of BM on central cholinergic systems

The expression of ChAT, a cholinergic marker in the hippocampus of animals was examined using WB. Compared with the sham-operated group, the vehicle-treated OBX group had a significantly reduced expression level of ChAT in the hippocampus (P<0.05). The OBX induced down-regulation of ChAT expression was reversed by BM and tacrine (P<0.05) (Fig 8).

In addition, acetylcholinesterase (AChE) activity *ex vivo* was measured in the cerebral cortex. As shown in Fig. 9A, there was no significant difference in the activity was observed between vehicle-treated sham and OBX groups. However, the activities of cortical AChE in the ATL- and

**Fig. 2** Effects of BM and THA administration on contextual and auditory (conditional) fear memory impaired by OBX. Each data column represents the mean ±S.E.M., indicated (n=8 -12). **P<0.01 vs. sham-operated group; # P<0.05, ## P<0.01 vs. water-treated OBX group.
tacrine-treated OBX groups were significantly reduced compared with that in the vehicle-treated OBX mice. On the other hand, treatment of cortical homogenates with tacrine showed a potent inhibitory effect on the in vitro activity of AChE with IC$_{50}$ = 21.8 ng/ml, while BM had a negligible effect on the activity with the IC$_{50}$ value was as great as 739.9 μg/ml (Fig 9B).

3. Discussion

In present study, we demonstrated that BM improves cognition deficits caused by OBX in mice, and suggested that the effect is at least in part due to facilitation of synaptic plasticity-related neuron signaling pathway including BDNF transcription and enhancement of cholinergic systems.

BM improves long-term memory deficits in OBX mice.

In this study, we found that administrations of BM and THA could attenuate OBX-induced deficits of long-term memory in the fear conditioning test (Fig. 2). It is well known that contextual memory and conditional memory elucidated in this test depend on the hippocampal function and the hippocampus plus amygdala function, respectively (25), and involve synaptic plasticity-related signaling and protein synthesis. Moreover, OBX reportedly induces impairment of fear responses to contextual and conditional auditory stimuli (26) as well as of the cholinergic systems.

Fig. 3  Effects of BM and THA treatment on expression levels of synaptic plasticity-related neuronal signaling proteins in the hippocampus. Each data column represents the mean ± S.E.M., indicated (n=5-6). *P<0.05, **P<0.01 vs. sham-operated group, # P<0.05, ## P<0.01 vs. water-treated OBX group.
in the hippocampus, cortex, and amygdala in rodents (17). Therefore, considering potential involvement of central cholinergic function in the amelioration of short-term memory deficit by BM, it is also very likely that improvement of OBX-induced long-term fear memory deficits by BM is at least mediated by the same mechanism(s) as BM-induced amelioration of short-term memory deficits of OBX animals.

Improvement of neuroplasticity-related signaling by BM administration

To obtain a better understanding, at the molecular level, of BM-induced improvement of cognitive dysfunction in OBX mice, we analyzed alterations of the expression levels of synaptic plasticity-related signaling proteins, a molecular biological feature of learning and memory. Evidence has demonstrated that the hippocampal glutamatergic neurotransmission is one of the molecular bases underlying learning and memory (27) and that stimulation of glutamate receptors triggers phosphorylation of some key proteins such as NMDAR, AMPAR, CaMKII and CREB and thereby is involved in synaptic plasticity in the hippocampus (28). Indeed, stimulation of AMPA- and NMDA-type glutamate receptors increases the intracellular Ca²⁺/Na⁺ level via the glutamate-gated cation channels on neuronal membranes and thereby activates calmodulin and other Ca²⁺-dependent enzymes. This signaling elicits the phosphorylation of subunits of NMDAR and AMPAR at the glutamatergic synapses via the activation of protein kinase C. Moreover, intracellular Ca²⁺-dependent activation of calmodulin triggers autophosphorylation of CaMKII and is reportedly implicated in the conversion of short-term memory to long-term memory. CREB, a nuclear transcription factors that can be activated by protein kinases A and/or C, also plays an important role in memory formation in a variety of cognitive tasks involving different brain structures (29). Its phosphorylated form, p-CREB, is implicated in the transcription of late downstream genes encoding proteins such as neurotrophic/growth factors including BDNF which plays a role in learning and memory (30, 31). In this study, we focused on these factors because of a couple of reasons.
First, OBX causes deterioration of the septo-hippocampal cholinergic system and thereby impairs learning and memory performance (12, 17). Second, this system can affect hippocampal glutamate receptor function via activation of muscarinic receptors coupled with Gq/11-PKC signaling systems and thereby modulate glutamatergic neurotransmission.

As shown in Figure 3, this study clearly revealed that OBX reduced levels of p-AMPAR, p-NMDAR1, p-CaMKII except p-CREB in the hippocampus without affecting basal expression levels of non-phosphorylated forms of these proteins, indicating that, in agreement with previous reports, OBX causes down-regulation of synaptic plasticity-related neuronal signaling systems. The present results that the CREB phosphorylation step analyzed in the hippocampus was apparently insusceptible to OBX agree with Han et al. (32). The reason for this different susceptibility to OBX is unclear but it may be due to a difference in the tissue area used for analysis.

It should be noted that phosphorylation of these neuronal signaling proteins except AMPAR in OBX animals could be reversed or even elevated in OBX groups treated repeatedly with BM and THA. These findings prompt us to consider that OBX-induced short-term and long-term cognitive deficits and their amelioration by THA and BM treatment are at least in part due to dysfunction and normalization of synaptic plasticity-related neuronal signaling systems, respectively, in the hippocampus. This hypothesis can be supported by changes in hippocampal expression of BDNF mRNA, one of important downstream factors of the signaling system, by THA and BM treatment of OBX animals, since OBX also down-regulated expression of BDNF mRNA in
the hippocampus in a manner reversible by THA and BM treatment (Fig. 4).

**Protection/facilitation of septo-hippocampal cholinergic systems by BM and THA**

Together with the present behavioral data that THA- and BM-induced amelioration of cognitive deficits caused by OBX was susceptible to a muscarinic receptor antagonist, facilitation of central cholinergic systems by THA and BM is likely to be involved in their ameliorating/enhancing effects on synaptic plasticity-related neuronal signaling system. This hypothesis seems substantiated by the findings that THA and BM administration attenuated down-regulated expression level of ChAT, a cholinergic nerve maker protein, caused by OBX. Indeed, both OBX-induced decrease in expression level of hippocampal ChAT and its reversal by THA observed in this study agree with previous reports (12, 13), indicating that OBX causes neurodegeneration of septo-hippocampal cholinergic systems and that THA protects septal cholinergic neurons from OBX-induced damage via endogenous acetylcholine. Therefore, it is of interest to note that BM administration could also attenuate OBX-induced down-regulation of ChAT expression in the hippocampus, suggesting protection of septal cholinergic neurons by BM from OBX damage (Fig. 5).

One of most plausible explanation for the mechanism underlying the effect of BM may be that BM administration facilitate function of central cholinergic systems by acting like THA, namely, inhibiting the activity of acetylcholinesterase in the brain. In fact, the present study
demonstrated that BM administration, like THA treatment, was capable of reducing ex vivo activity of cholinesterase in the brain tissues and that BM extract itself, like THA, has the potential to inhibit in vitro cholinesterase activity of brain homogenates (Fig. 6). However, the inhibitory effects of BM on ex vivo and in vitro activities of cholinesterase were clearly less potent than THA even at a dose which exhibited the same extents of behavioral and neurochemical improvements as THA. These findings lead us to suggestion that BM-induced improvement of cognitive dysfunction in OBX mice is mainly mediated by mechanisms differing from cholinesterase inhibition.

Conclusion

Present study has demonstrated that daily administration of BM ameliorates cognition impairment of OBX mice and that facilitation of LTP-related neuronal signaling and BDNF via stimulation of cholinergic systems are involved in the action of BM

Acknowledgements

This work was in part supported by a Grant-in-Aid for the 2010 and 2012 Cooperative Research Project II from the Institute of Natural Medicine, University of Toyama (to H.T.N.P. and K.M.). Do Thi Phuong (NIMM, Hanoi, Vietnam) and Le Thi Xoan (NIM, Toyama, Japan) have contributed to collection and analysis of the data in this collaboration project.

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