Improvement of cognitive deficits in SAMP8 mice by 3-n-butylphthalide

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The herbal extract 3-n-butylphthalide (NBP) is used in clinical practice for ischemic patients in China. It has been shown to have various neuroprotective effects both in vitro and in vivo. In the present study, the effects of NBP on learning and memory decline in the senescence-accelerated mouse prone-8 (SAMP8) animal model were investigated. Intragastric NBP administration to 4-month-old SAMP8 mice for 2 months significantly improved spatial learning and memory ability. Moreover, the loss of choline acetyltransferase (ChAT)-positive neurons in the medial septal nucleus and the vertical limb of the diagonal band in SAMP8 mice was slowed down, as was the decline in the protein and mRNA expression of ChAT in the hippocampus, cerebral cortex, and forebrain. These results demonstrated that NBP treatment starting at the age of 4 months protected from the learning/memory deficits with aging of SAMP8 mice, and that this effect might be mediated by preventing the decline of the central cholinergic system.

Keywords: 3-n-Butylphthalide, Learning and memory deficits, Choline acetyltransferase, SAMP8

Abbreviations
NBP: 3-n-butylphthalide
SAM: senescence-accelerated mouse
SAMP8: senescence-accelerated mouse prone-8
SAMR1: senescence-accelerated mouse-resistant/1
ChAT: choline acetyltransferase
AD: Alzheimer’s disease
ABC: avidin–biotin complex
PBS: phosphate buffered saline
BSA: bovine serum albumin
DAB: 3,3′-diaminobenzidine
PMSF: phenylmethylsulfonyl fluoride (protease inhibitor)
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
TPBS: tris phosphate buffered saline
APP: amyloid beta precursor protein
MMLV: Moloney murine leukemia virus
NMDA: N-methyl-D-aspartate
aFGF: acidic fibroblast growth factor

Introduction
Aging-related diseases associated with progressive loss of learning and memory function have become an important medical and social problem due to the increasing number of elderly individuals.¹ Thus, developing appropriate diagnostic criteria and drugs for early cognitive impairment is important to public health.

Currently, no available treatments have been shown to unequivocally reverse existing learning and memory deficits or to arrest disease progression in aging individuals. One of the important symptoms of Alzheimer’s disease (AD) is cognitive impairment. It has been reported that cholinergic neuronal pathways is degenerated and choline acetyltransferase (ChAT) is depleted in AD, thus therapies that aims at improving cholinergic system function have been used in an effort to provide symptomatic treatment for cognitive dysfunction.²

3-n-Butylphthalide (NBP), a small molecule drug derived from a Chinese herb, is being used in clinical practice for ischemic patients in China. It has been found to attenuate the learning and memory deficits induced by chronic cerebral hypoperfusion in rats.³ Most of the positive effects of NBP, such as improved microcirculation in pial arterioles,⁴ decreased volume of cerebral infarct,⁵ ameliorated mitochondrial dysfunction,⁶ decreased oxidative damage,⁷ reduced neuronal apoptosis⁸ and inhibition of the inflammatory response,⁹ have been verified in ischemic patients and the vascular dementia mouse model.¹⁰ Even though the specific target of NBP is not clear yet, previous studies suggest that it may work with multiple targets and provide neuron protection as well as improve cerebral blood flow. Given the benefits of NBP treatment, we believe further research on the role of NBP in learning and cognition should have far-reaching significance.
The senescence-accelerated mouse (SAM)-pron/8 (SAMP8) and the senescence-accelerated mouse-resistant/1 (SAMR1) mouse strains were established by Takeda’s group in the early 1970s, and are widely used as a model of aging. Whereas the SAMP8 strain spontaneously develops learning and memory deficits during its aging process, along with various pathological signs of neurodegeneration, the SAMR1 strain shows normal brain aging, and this permits its use as a control.

Numerous studies using SAMP8 model have been carried out in various fields of aging study since the first paper on SAM development was published in 1981. We have proved that the rapid learning and memory deficits that occur along with aging in SAMP8 mice are similar to those found in other aged animals. It has been accepted that the number of cholinergic neurons in the medial septum apparently reduce with aging, which is related to impaired cognition abilities in SAMP8 mice. It has also been suggested that the function of cholinergic neurons is involved in learning and memory, and the age-dependent decline of this function is correlated with a decrease in cognitive performance of SAMP8 mice. Based on these findings, a more systematic study on ChAT expression in SAMP8 mice would be quite important and necessary.

In this study, we investigated the protective effects of NBP on early cognitive impairment of SAMP8 mice, and explored the possible mechanisms underlying the prevention of age-related learning and memory deficits in SAMP8 mice.

Materials and Methods

Experimental animals and drug

Four-month old male SAMP8 mice and SAMR1 mice were obtained from the pathogen-free facility of the Experimental Animal Center of the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. The mice were housed in standard cages at the unit of neural degenerative diseases, the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. According to previous studies, the tested SAMP8 mice were treated either with a vehicle of 5% Tween-80 (Pcon), or different doses of NBP, such as 40 mg/kg NBP (P40), 80 mg/kg NBP (P80), and 160 mg/kg NBP (P160). In each point we used 15 animals. Fifteen SAMR1 mice were also chosen for control group (no treatment) (Rcon).

The NBP used in our trial was supplied by Shijiazhuang Pharmaceutical Group NBP Pharmaceutical Co., Ltd (Batch No. M001-070427-01). The NBP was given orally daily by a gavage needle after being made into an emulsion with 5% Tween-80. The NBP treatments were continuously performed for 60 days. All the experimental procedures were formally approved by the Animal Subjects Review Board of the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Morris water maze test

After various treatments, Morris water maze tests were carried out after 1 week of acclimatization in the testing room. The water maze was a circular tank with a diameter of 100 cm and height of 50 cm, made of wood and painted black. The tank was filled with a mixture of cold and hot tap water to a depth of 0.3 m. The water temperature was maintained at 23 ± 2°C from the beginning of each testing day. Yellow curtains were drawn around the pool (50 cm from the pool periphery). Distinctive visual marks were attached on the curtains, which served as distal cues. Small black plastic granules were added to make water opaque. The experiments were designed as double blind experiments. The animals were coded and blind to experimenter. The animals coding information was also blind to the personnel who performed data analysis.

A single habituation trial was performed 1 day before the first day of the water maze memory test. The detailed procedures for learning and memory task of the Morris water maze were similar to those described in our previous study. The escape latency time was recorded to assess learning ability. The crossing times over the platform and the retention time in the probe trial were recorded to evaluate memory ability.

Immunohistochemical staining

After NBP treatments, mice (n = 5 per group) were given a lethal intraperitoneal dose of sodium pentobarbital (2 mg/10 g body weight). They were then perfused via the ascending aorta with 10 ml of saline followed by 100 ml of 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.2). A pump was used to control flow rate (20 ml/min). The brains were carefully removed and fixed in the same fixative overnight, and then transferred to 30% sucrose for an additional 24 hours. The forebrain block was dissected on a Rodent Brain Matrix and then sectioned (40 μm) with a sliding microtome (Leica Microsystems, GmbH, Wetzlar, Germany). The medial septum was analyzed in its entirety by sectioning the brain beginning from the appearance of the genu of the corpus callosum to the decussation of the anterior commissure (bregma level 1.18–0.14 mm). The staining protocol employed a modified avidin–biotin complex (ABC) immunohistochemistry procedure, which has been described previously. Sections were pretreated with 0.05 M phosphate buffered saline (PBS, pH 7.2) containing 40% methanol and 10% hydrogen peroxide for 30 minutes, and then incubated with primary polyclonal rabbit
ChAT antibody (1:1000, Chemicon, Temecula, CA, USA) in PBS containing 1% BSA (bovine serum albumin) and 0.01% Triton X-100 overnight at room temperature. Sections were incubated with goat-anti-rabbit IgG (1:200, Chemicon) in PBS containing 1% BSA and 0.01% Triton X-100 for 2 hours after rinsing in PBS, and then incubated in avidin–biotin–peroxidase complex for another 2 hours (1:1000 in PBS containing 1% BSA and 0.01% Triton X-100), rinsed in PBS and developed in 0.05% 3,3’-diaminobenzidine (DAB) and 0.003% H2O2 in PBS. The medial septum was bilaterally examined, and the results of morphometric analysis were reported as total cell counts for structures on both sides.25,26 Three sections across the medial septum of five mice in each group were counted (n=15 sections per group), and the average count for each group was recorded (cells/mm²). The ChAT-positive neurons were captured by a video monitor using an Olympus microscope 10× objective, and then processed by NIH Image software. The final figures were generated using Adobe PhotoShop CS and Adobe Illustrator CS software.

Western blot
After the last behavior test, the mice (n=10 per group) were deeply anesthetized by giving a lethal intraperitoneal dose of 10% chloralalurad (2 mg/10 g body weight). In every mouse, 10 ml of 0.9% sodium chloride was perfused via the ascending aorta. The brains were removed and three regions were dissected bilaterally examined, and the results of morphometric analysis were reported as total cell counts for structures on both sides.25,26 Three sections across the medial septum of five mice in each group were counted (n=15 sections per group), and the average count for each group was recorded (cells/mm²). The ChAT-positive neurons were captured by a video monitor using an Olympus microscope 10× objective, and then processed by NIH Image software. The final figures were generated using Adobe PhotoShop CS and Adobe Illustrator CS software.

During the tests, no significant body weight changes were observed with each of the treated animals (P>0.05). The escape latency time for each group of treated animals are shown in Fig. 1A. It is clearly demonstrated that all SAM mice performance were significantly improved over the

Statistical analysis
All the above experiments were designed as double blinded. Data were presented as mean±standard deviation (SD) and the alterations were analyzed by analysis of variance (ANOVA). A difference with P<0.05 was considered statistically significant. In cases where a significant difference was detected, a specific post hoc comparison between groups was examined with Student–Newman–Keuls tests (compare all pairs of groups) or Dunnett’s T3 tests (compare all other groups vs one group). The analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). The density of immunoblotting was quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The ratio of ChAT to GAPDH (ChAT/GAPDH) and amyloid beta precursor protein (APP) to GAPDH (APP/GAPDH) represented the level of protein expression.

PCR test
Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using Oligo(dT)18, dNTP, and Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara, Japan). Reverse transcription PCR (RT-PCR) was performed to examine the expression of ChAT with gene-specific primers for ChAT designed by using Primer 5.0. The sequences of the ChAT DNA oligos were 5’-GGTGCCCCAGAGGCGATATC-3’ and 5’-ATTGGAGCAGGC-CTTCTATC-3’. Primers for a house-keeping gene (beta-actin) were designed as an internal control. The sense and antisense primers of beta-actin were 5’-CCTGTCGCCCAACAGTGTC-3’ and 5’-ATACTCTGTGCTTGCAGTCC-3’. PCR consisted of an initial denaturation at 95°C for 30 minutes, followed by 25 cycles of 10-second denaturation, 20-second annealing at 57°C and 20-second extension at 72°C. PCR products were detected by electrophoresis on 1.8% agarose gels. In order to validate the gene differential expression identified by PCR, the mRNA level of ChAT was detected using quantitative real-time PCR (qPCR) with the same primers for ChAT and the house-keeping gene beta-actin. 

Results
3-n-Butylphthalide improves learning and memory deficits in SAMP8 mice
After different treatments, Morris water maze tests were carried out after 1 week of acclimatization in the testing room. During the tests, no significant body weight changes have been observed with each of the treated animals (P>0.05). The escape latency time for each group of treated animals are shown in Fig. 1A. It is clearly demonstrated that all SAM mice performance were significantly improved over the
first five training days and the following relearning days. Meanwhile, the swimming speed in every group recorded during the whole trial showed no significant difference (Fig. 1B). There was no significant difference in escape latency time among the five groups on Day 1 (\(P > 0.05\)). The escape latency time of the Rcon group was significantly decreased on Day 2 compared with the Pcon group (18.5 ± 8.1 seconds in Rcon vs 49.9 ± 13.7 seconds in Pcon, \(P < 0.001\)). From Day 2 to Day 5, the escape latency time of NBP-treated groups were significantly shortened than that of the Pcon group. Further comparison with the Rcon group showed that the escape latency time of the P40 group was significantly longer (P40 vs Rcon on Day 2, \(P < 0.01\); P40 vs Rcon on Day 5, \(P < 0.05\)), while the escape latency times of the P80 and P160 groups were not significantly different from that of the Rcon group (P80, P160 vs Rcon on Day 2, \(P > 0.05\); P80, P160 vs Rcon on Day 5, \(P > 0.05\)). A reversal trial from Day 7 to Day 11 was carried out to identify the relearning ability and flexibility of SAM mice. The escape latency time of the P160 and Rcon group recorded on Day 7 was significantly shorter than that of the Pcon group, and that of the Pcon group had no significant changes during the reversal trial. The escape latency time was much longer in the Pcon group compared with the Rcon group on Day 11 (\(P < 0.001\)), while that of the NBP-treated groups was not significantly different compared with the Rcon group (P40, P80, P160 vs Rcon, \(P > 0.05\)). The tests showed that when compared to SAMR1 mice, SAMP8 mice had spatial learning deficits and that NBP treatments could improve SAMP8 mice learning deficits.

A probe trial on Day 6 was made to identify the memory function of SAM mice. One important refined parameter for the spatial bias is the number of crossings over to the former location of the platform.\(^{28}\) The result shown in Fig. 1C indicated that NBP-treated groups crossed to the correct site significantly more often than the Pcon group did (\(P < 0.001\)). Meanwhile, the Rcon group spent much more time in the former platform quarter compared with the Pcon group (25.5 ± 8.8 seconds in Rcon vs 8.6 ± 3.3 seconds in Pcon, \(P < 0.001\)), as did the P40, P80 and P160 groups compared with the Pcon group (15.7 ± 7.8 seconds in P40, 16.6 ± 4.8 seconds in P80, 22.5 ± 5.3 seconds in P160 vs 8.6 ± 3.3 seconds in Pcon, \(P < 0.05\), \(P < 0.01\), \(P < 0.001\)), which means the SAMR1 and NBP-treated mice had better
memory of the former platform location (Fig. 1D). The visible platform trial on Day 12 excluded the impact of vision in the different groups. Shorter escape latency time in the learning process, more crossings and longer time in the target quadrant in the memory test were noted in the NBP groups and SAMR1 group.

3-\textit{N}-Butylphthalide protects the basal forebrain cholinergic pathway

The central cholinergic system is crucial for spatial learning and memory in rodents. In our study, the change of cholinergic neurons in the medial septum was investigated by immunoreactivity of ChAT. The average number of ChAT-positive neurons in the Pcon group (Fig. 2A) was 44.1 ± 14.6 cells/mm², and the Rcon group (Fig. 2B) is 93.3 ± 7.5 cells/mm² ($P < 0.001$). The number was increased by 36.87, 58.14, and 68.78%, respectively, in the P40 group ($60.7 \pm 15.3$ cells/mm², $P < 0.01$), P80 group ($69.9 \pm 13.5$ cells/mm², $P < 0.001$), and P160 group ($74.3 \pm 15.8$ cells/mm², $P < 0.001$) compared with the Pcon group. At the age of 6 months, the number of ChAT-positive neurons in the septal region of the Pcon group rats was 50% less than that of the Rcon group (Fig. 2C).

Effects of NBP on ChAT expression

The hippocampus, cerebral cortex, and forebrain are three important regions of the brain for studying learning and memory abilities. Here is a systemic observation of ChAT expression in SAM mice in these brain areas.

Positive ChAT PCR products (180 bp) were observed in each group and were most abundant in the Rcon group and least abundant in the Pcon group (Fig. 3). In NBP-treated groups the mean levels of ChAT mRNA increased and were significantly higher than those in the Pcon group in all three regions. Quantitative real-time PCR was also performed and showed a decline of ChAT abundance in the Pcon group that was significantly lower by nearly 3.5, 3.1 and 5.2-fold relative to the Rcon group in three regions, respectively. The ChAT mRNA level in the Pcon group was consistently significantly decreased compared with the Rcon group, while NBP treatment increased the abundance of ChAT. In the hippocampus, the mean level of ChAT mRNA in the P40, P80 and P160 groups was approximately 1.5, 1.7 and 1.9-fold higher, respectively, relative to that in the Pcon group (Fig. 3A). An increased expression of ChAT mRNA by nearly 1.5, 1.7 and 2.2-fold, respectively, was observed in the cerebral cortex (Fig. 3B) and 2.2, 1.8, and 3.6-fold, respectively, in the forebrain (Fig. 3C).

Western blotting was performed to determine the protein level of ChAT in the hippocampus (Fig. 4A), cerebral cortex (Fig. 4B) and forebrain (Fig. 4C). The abundance of ChAT in all three regions decreased visibly in the Pcon group compared with the Rcon group, and was significantly elevated in the NBP-treated groups. The ChAT protein in the Pcon group was reduced by almost 4-fold relative to that in the Rcon group in the three regions. In the NBP-treated groups, the relative expression of ChAT in the P160 group was nearly 2.8, 3.3 and 2.2-fold higher than in the Pcon group in the three regions, respectively. Combined with the mRNA level of ChAT, the abundance of ChAT expression decreased with aging in the SAMP8 mice, while NBP improved the ChAT level.

Discussion

With advancing age, humans and animals undergo gradual decline of learning and memory. Such age-related change is the most important risk factor for learning- and memory-related diseases such as AD. Previous studies have shown evidence of changes that might have effects on the pathology of age-related diseases such as death of neurons, loss of synapses, cholinergic deficits, inflammatory processes and impaired learning and memory. In order to study learning and memory impairment in the late period of animal’s life span, many animal models have been established. Senescence-accelerated mouse has been established as a murine model of accelerated aging. The SAMP8 strain, one of the most popularly used animal models for studying early learning and memory problems, shows age-related deterioration of learning and memory at an earlier age. Actually, our results provide further evidence that impairments of learning and memory in vehicle treated SAMP8 mice compared with SAMR1 mice (Fig. 1A), which is consistent with previous reports.

A number of studies have shown that SAMP8 has cholinergic deficits, oxidative damage, alterations in membrane lipids and circadian rhythm disturbances. The other characteristics of AD shared by SAMP8 mice are among others, hyperphosphorylation of Tau, an increase in presenilin, increased glutamate, altered N-methyl-D-aspartate (NMDA) function and increased neuronal nitric oxide synthase. Thus, many AD features can be studied with the SAMP8 mouse model.

One of the prominent characters of AD is a widespread degeneration of the basal forebrain acetylcholinergic system. The biosynthesis of acetylcholine is catalyzed by ChAT, an enzyme that is characteristically reduced in AD to an extent that correlates with the severity of dementia. Cholinergic system in the brain plays an important role in age-related decline of learning and memory. Choline acetyltransferase is presently the most specific
indicator for monitoring the functional state of cholinergic neurons. Choline acetyltransferase-positive neuron loss is a hallmark of pathological feature. The cholinergic neurons in the medial septal reduced apparently with aging and it was related to impaired cognition abilities in SAMP8 mice. On the other hand, several studies also demonstrated that SAMP8 mice showed less ChAT activity, decreased NMDA-evoked Ach release and lower binding of antagonists to muscarinic receptors. In addition, the neuronal dysfunction seems to underlie the disturbances of learning and memory. It has been suggested that a dysfunction of septal cholinergic neurons exists in terms of their ability to produce
ChAT. Cholinergic neurons in the medial septum send their axons to the hippocampus and comprise the septohippocampal pathway. Thus, in SAMP8 mice, a dysfunction of the septal cholinergic neurons induces a functional disturbance of the septohippocampal pathway, leading to impairments of learning and memory.18,23 Yet, it is more important to find ways of treating cognitive disorders associated with aging. Recently, many efforts have been focused on neuronal protection and improvement of learning and memory deficit in aged animals. Some studies have focused on using anti-oxidants, such as acetyl-L-carnitine, alpha-lipoic acid, and ginsenoside38,39 while some focused on improving mitochondrial function.16 However, probably more studies have tried to treat learning and memory deficit by improving acetylcholinergic system degeneration. Previous reports showed that injecting acidic fibroblast growth factor (aFGF) could improve learning and memory deficit in SAMP8 mice by a preservation of function in medial septum cholinergic neurons.23 Harold et al. suggested using acetylcholinesterase inhibitors in AD to exert their therapeutic effects by at least partially and temporarily reversing the cholinergic deficit by preventing the degradation of synaptic acetylcholine.35

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Researchers also tried using neurotrophic factors to treat neurodegenerative disorders. Matsui et al. found that the age-related cholinergic deficits were prevented by treatment with either magnolol or honokiol, small organic compounds with neurotrophic activity derived from the Magnolia plant. In this study, we found that the decline of the central cholinergic system occurring in SAMP8 mice with aging was confirmed, and the decrease of ChAT was correlated with cognitive impairments. We further discovered that NBP, an extract from seeds of Apium graveolens Linn (Chinese celery), can markedly improve cognitive deficits in SAMP8 mice (Fig. 1C and D). Such phenomenon is quite similar to what we observed in previous studies. We also found that NBP treatment not only prevented decline in the
learning and memory ability in SAMP8 mice, but also prevented the decline of the central cholinergic system by checking mRNA level to protein expression level (Figs. 2–4). In previous AD studies, progressive cholinergic denervation as well as decreased levels of ChAT has been reported to be associated with AD-relevant cognitive impairment, and dysfunction in the cholinergic system of the basal forebrain has been found to be involved in the pathogenesis of AD. Thus, NBP may have the potential therapeutic applications to correct AD neurodegenerative disorders.

Although NBP’s target in the central nervous system is not clear yet, other studies show that NBP can improve cognitive deficits in APP/PS1-Alzheimer’s transgenic mice by reducing tau phosphorylation, suggesting that NBP may serve as a promising candidate of multi-target neuronal protective agent for the treatment of Alzheimer’s disease. NBP was also reported to reduce the cerebral infarct volume in the transient cerebral ischemia rats. In conclusion, our findings in this study have confirmed the behavioral changes with aging in SAMP8 mice, and demonstrated that NBP treatment might provide a promising avenue for preventing or ameliorating age-related disorders.

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