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Enhanced Learning of Normal Adult Rodents by Repeated Oral Administration of Soybean Transphosphatidylated Phosphatidylserine

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Abstract. Soybean lecithin transphosphatidylated phosphatidylserine (SB-tPS) is already known to improve the learning ability of aged or drug-induced amnesic rodents. In this study, its effect on normal adult rodents was evaluated using several learning tasks. Firstly, three behavioral tests (open-field, Y-maze, and active avoidance test) were consecutively carried out after the daily oral administration of SB-tPS (50 mg/kg per day, for 34 days). Repeated oral administration of SB-tPS did not affect either exploratory behavior in the open-field test or spontaneous alternation behavior in the Y-maze test, while mice pretreated with SB-tPS showed significant enhancement of conditioned avoidance response. Secondly, the brightness discrimination test was used to evaluate the effect of SB-tPS on learning ability. The daily oral administration of SB-tPS (50 mg/kg per day, for 27 days) to normal rats significantly increased the correct response ratio in the brightness discrimination test. Finally, to elucidate the necessity of SB-tPS pretreatment, another active avoidance test was carried out, and no enhancement of conditioned avoidance response was observed in non-pretreated mice. These results suggest that repeated administration of SB-tPS could enhance the learning ability of normal adult rodents as those of aged ones.

Keywords: phosphatidylserine, learning, normal adult rodent, active avoidance, brightness discrimination

Introduction

Phosphatidylserine (PS) is a constituent of biological membranes, abundant in the brain, and is known to exert several effects on the central nervous system, particularly regarding amnesia (1). The attenuating effects of PS on memory impairment associated with Alzheimer's disease or aging have been demonstrated in several clinical studies (2 – 6). In these clinical studies, PS extracted from bovine cortex (BC-PS) was used; however, the use of BC-PS as a foodstuff has been limited because of the risk of contamination by the prion, which is likely to cause bovine spongiform encephalopathy (7).

Our group developed a method for producing PS from soybean lecithin and L-serine by the transphosphatidylation reaction of phospholipase D; and PS produced by this method, which we call "soybean lecithin transphosphatidylated PS (SB-tPS)", was shown to ameliorate the drug-induced memory impairment in rodents similar to BC-PS (8 – 10). Recently, our group also showed that the oral administration of SB-tPS for 60 days improved spatial memory impairment in aged rats (11). Furthermore, SB-tPS has also been demonstrated to prevent ischemic damage to the brain in gerbils (12).

There are many reports showing that BC-PS improves learning ability in aged or drug-induced amnesic animals (13 – 15) and the mice during postnatal development (16, 17), but there are few reports on normal adult animals. Only one report (18) showed a positive effect of BC-PC on normal adult rats. In this report, rats were divided into two groups by their avoidance learning capability and the effect of BC-PS (15 mg/kg per day, i.p., for 30 days) on the avoidance learning was evaluated in both groups. BC-PS administration in-

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creased the avoidance performance only in poor performing rats without changing that in those performing well. Thus, it remains unclear whether PS can enhance the learning ability of normal adult animals.

In this study, the nootropic effects of orally administered SB-tPS on normal adult rodents were determined using the open-field test, Y-maze test, active avoidance test, and brightness discrimination test.

Materials and Methods

Animals

Male ddY mice (8- and 12-week-old; Nihon SLC, Shizuoka) were used for experiments 1 and 3. The mice were given free access to food and water. In experiment 2, male Fischer 344 rats (8-week-old, Nihon SLC) were used for brightness discrimination tests after acclimatization to handling and food pellets. The rats were maintained at 85% of their free-feeding weight by restricted feeding throughout the experiment. All animals were housed under standard conditions (22 ± 2°C, 50 ± 10% humidity, 12-h light-dark cycle with lights on at 8:00 a.m.). The care and treatment of the animals complied with the guidelines for the ethical treatment of laboratory animals at Meijo University.

Materials

SB-tPS was prepared from soybean phosphatidylcholine and L-serine by transphosphatidylation using phospholipase D as reported previously (8). SB-tPS emulsified by sonication in isotonic saline was orally administered (50 mg/kg) with a probe according to each experimental schedule. Isotonic saline was administered as a control. All behavioral tests were begun 1 h after administration.

Experiment 1

The open-field test, Y-maze test, and active avoidance test were consecutively conducted on the 35th, 36th, and 41st to 56th day from the beginning of SB-tPS treatment, respectively, as shown in Fig. 1. This experiment used 8-week-old ddY mice.

Open-field test: A white Plexiglas box with the floor (40 × 40 cm) divided into 25 squares with black lines was used. Each mouse was put in the corner of the box and its movements (ambulation, rearing, line-crossing, grooming, and defecation) were recorded for 5 min.

Y-maze test: A black Y-maze made of plywood was used. Each arm was 40-cm-long, 12-cm-high, 3-cm-wide at the bottom and 10-cm-wide at the top and positioned at an equal angle. The testing procedure was the same as in the previous report (19). Each mouse was placed at the end of one arm and was allowed to move freely through the maze for a 8-min test session. The sequence of arm entries was recorded manually. An alternation was defined as an entry into all three arms on consecutive occasions. The number of maximum alternations was therefore the total number of arms entered minus 2, and the percent alternation was calculated as (actual alternations / maximum alternations) × 100.

Active avoidance test: A skinner box (18 × 15 × 18 cm; MED, St. Albans, VT, USA) was used. On the front wall, a cue-lamp was mounted above a stainless steel lever. The floor was made of stainless steel bars

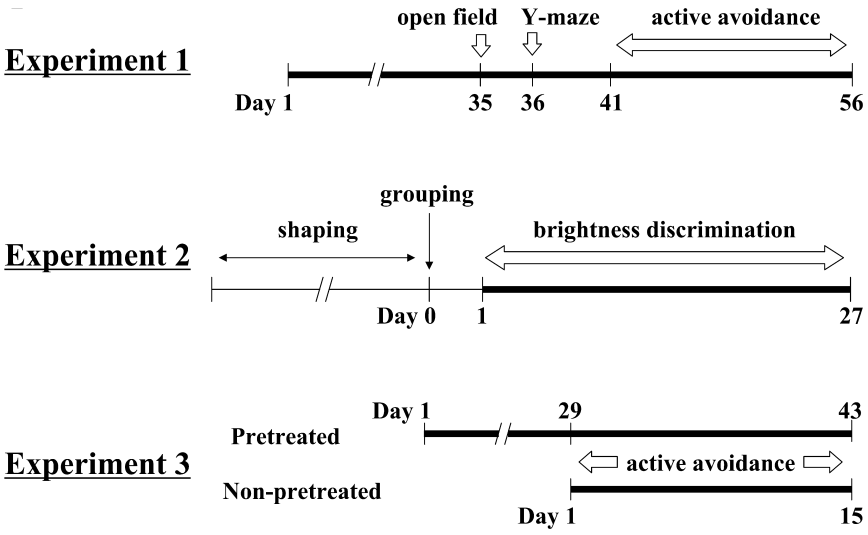

Fig. 1. Experimental schedule of three experiments in this study. Bold line indicates the period of SB-tPS or saline administration.

for electrical shocks, and a buzzer was installed. All equipment in the box was controlled with a computer system (MED-PC; Neuroscience, Tokyo). A session of 60 trials per day was conducted for 16 consecutive days. A 1-min trial consisted of a 40-s intertrial interval (ITI), a 10-s presentation of the conditioned stimulus (CS) with the cue lamp and buzzer, and a 10-s presentation of unconditioned stimulus (UCS) with the cue lamp, buzzer, and scrambled electrical shocks (0.3 mA). When the mouse pushed the lever during the presentation of CS or UCS, the trial was cancelled and the next trial was begun. The percentage of trials in which an electrical shock was avoided (i.e., the mouse pushed the lever during the presentation of CS) was defined as a learning index (20). The number of lever presses in ITI was also counted.

Experiment 2

A brightness discrimination test was performed using 8-week-old rats as shown in Fig. 1. A one-lever operation chamber ($30 \times 25 \times 27$ cm; Muromachi Kikai, Tokyo) with some modifications was used. Its bank of three white cue lamps mounted above the response lever on the front wall was covered with a half cylindrical piece of white translucent plastic to make it look like one lamp. A food magazine, which delivered a 45-mg food pellet, was also mounted on the wall. The experimental procedures were described in a previous report (21). Brightness discrimination was carried out at a variable interval of 15 s (VI-15) after shaping the animals. When the brighter light (all three lamps inside the half cylindrical cover turned on) was presented as a positive stimulus (S^+) for 20 s, the lever-pressing responses were reinforced by food pellets at VI-15. In contrast, when the dimmer light (S^-, only one center lamp turned on) was on, the food pellet was not given for any response with the lever. One session consisted of 20 rounds each of S^+ and S^- (20 S^+ and 20 S^-) in a random order, and the session was run daily for 27 consecutive days. The correct response ratio, $R^+ / (R^+ + R^-) \times 100$, was calculated from the number of correct lever-pressing responses (R^+) during the S^+ presentation and that of incorrect responses (R^-) during the S^- presentation in one session. After the shaping stage of the brightness discrimination test, rats were divided into two groups and SB-tPS administration was started. Since the rats were trained to press a lever to receive a food pellet at the shaping stage, individual differences in response at the start of brightness discrimination can be excluded.

Experiment 3

Another active avoidance test was conducted using 8- and 12-week-old mice as shown in Fig. 1. In 8-week-old mice, SB-tPS was administered for 4 weeks before the test, and therefore active avoidance was started at 12 weeks of age. In 12-week-old mice, SB-tPS was administered only during the 15-day test period. The procedure was as described in Experiment 1.

Statistical analyses

Regarding the parameters in the open-field test and the Y-maze test, significant differences were evaluated by Student's *t*-test. For active avoidance and brightness discrimination, differences between the two groups were determined with 2-way ANOVA, and the difference of each session between the two groups was analyzed by the Wilcoxon rank-sum test. $P<0.05$ was considered significant.

Results

Experiment 1

Open-field test and Y-maze test: Table 1 shows the performances in the open-field and the Y-maze test. In the open-field test, there were no significant differences in any parameters between the control and SB-tPS group. SB-tPS did not affect percent alternation or the total number of arm entries in the Y-maze test.

Table 1. Effects of oral administration of SB-tPS in the open-field test and Y-maze test

		Control	SB-tPS
Open-field test	Ambulation	140.8 ± 12.9	160.0 ± 12.7
	Rearing	33.6 ± 3.5	34.9 ± 2.2
	Grooming (s)	14.8 ± 2.6	11.3 ± 2.1
	Feces	3.5 ± 0.4	2.9 ± 0.3
Y-maze test	Total arm entries	21.6 ± 1.0	23.8 ± 1.6
	% Alternation	72.2 ± 2.4	70.7 ± 2.8

SB-tPS (50 mg/kg, p.o.) was administered for 34 days before the open-field test, and the Y-maze test was performed on the day after the open-field test. Data represent the mean ± S.E.M. (n = 20). No difference was detected between the control and SB-tPS (Student's *t*-test).

Active avoidance: Although the avoidance rates of both groups gradually increased in the early session, the rate of the control group showed little change after session 7 (Fig. 2A). There was a significant difference between the control and the SB-tPS group ($F[1, 608] = 17.1$, $P<0.01$, 2-way ANOVA), and post hoc analysis showed significant differences in each session after session 10 ($P<0.05$, Wilcoxon rank-sum test). The number of lever presses in ITI was significantly fewer in the SB-tPS group than the control ($F[1, 608] = 4.39$, $P<0.05$, 2-way ANOVA) (data not shown).

In the early session (sessions 1 to 7), about one third of the mice in both groups seldom avoided the electrical shock. Therefore, we defined mice whose avoidance rate was below 10% in every early session from 1 to 7 as the low responding (LR) group and the rest of mice as the high responding (HR) group (Fig. 2B). In the LR group, 3 out of the 7 SB-tPS-treated mice began to avoid the electrical shock in the later session, while none of the control mice could avoid the electrical shock throughout the test period ($F[1, 192] = 17.8$, $P<0.01$, 2-way ANOVA). Significant improvement ($F[1, 384] = 20.5$, $P<0.01$, 2-way ANOVA) was also shown in the HR group.

Experiment 2

The correct response ratios in brightness discrimination are shown in Fig. 3A. A significant effect of SB-tPS on the control was revealed by 2-way ANOVA ($F[1, 486] = 7.14$, $P<0.01$), although the significant difference was not detected in each session (Wilcoxon rank-sum test). When the brightness discrimination period was divided into three (the first 9, middle 9, and last 9 sessions), statistical significance was only detected in the last 9 sessions ($F[1, 162] = 7.19$, $P<0.01$, 2-way ANOVA). The number of lever presses in the brightness discrimination is shown in Fig. 3B. The correct response number did not differ between the two groups ($F[1, 486] = 0.84$, $P>0.05$, 2-way ANOVA), while the incorrect responses were significantly fewer in the SB-tPS group than in the control group ($F[1, 486] = 8.82$, $P<0.01$, 2-way ANOVA).

Experiment 3

When SB-tPS was administered for 4 weeks before the beginning of the test, the avoidance rate significantly increased (Fig. 4A; $F[1, 570] = 8.47$, $P<0.01$, 2-way ANOVA). In contrast, when SB-tPS was administered only during the 15-day test period, SB-tPS was without any significant effects (Fig. 4B; $F[1, 570] = 0.359$, $P>0.05$, 2-way ANOVA). In this experiment, no

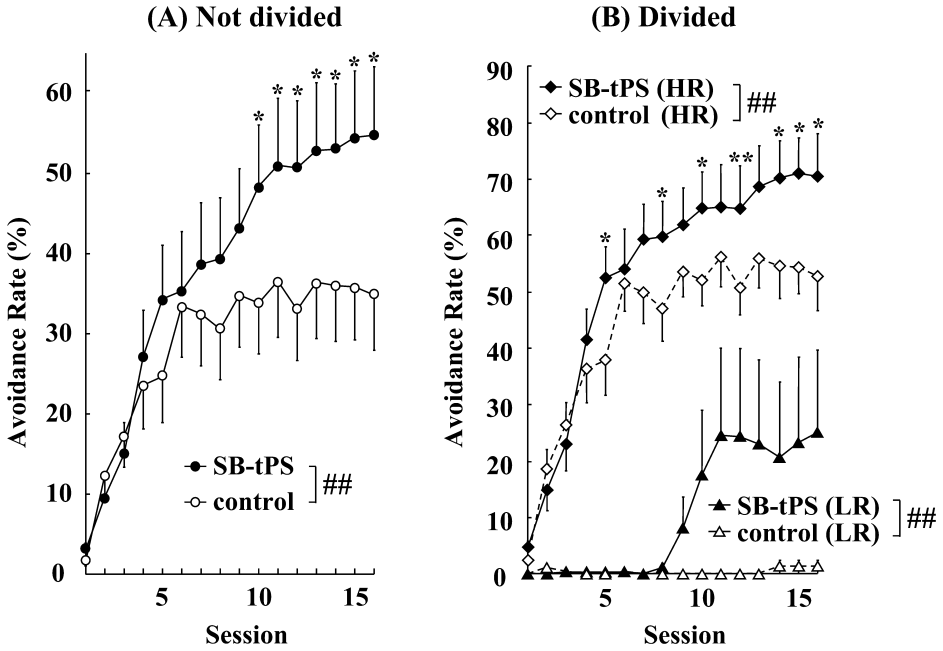

Fig. 2. Effect of the oral administration of SB-tPS (50 mg/kg) on the active avoidance test. The test was started on the 41st day from the beginning of SB-tPS administration. A: Data were not divided (n = 20). B: Data were divided into two groups (LR: low responding, HR: high responding) by their performance level between sessions 1 and 7 (LR: n = 7, HR: n = 13). Data represent the mean ± S.E.M. Comparisons between two groups in the total sessions (session 1 to 16) were carried out by 2-way ANOVA. $^{\#\#}P<0.01$. Post hoc comparisons between two groups in each session were carried out by the Wilcoxon rank-sum test. $^{*}P<0.05$, $^{**}P<0.01$.

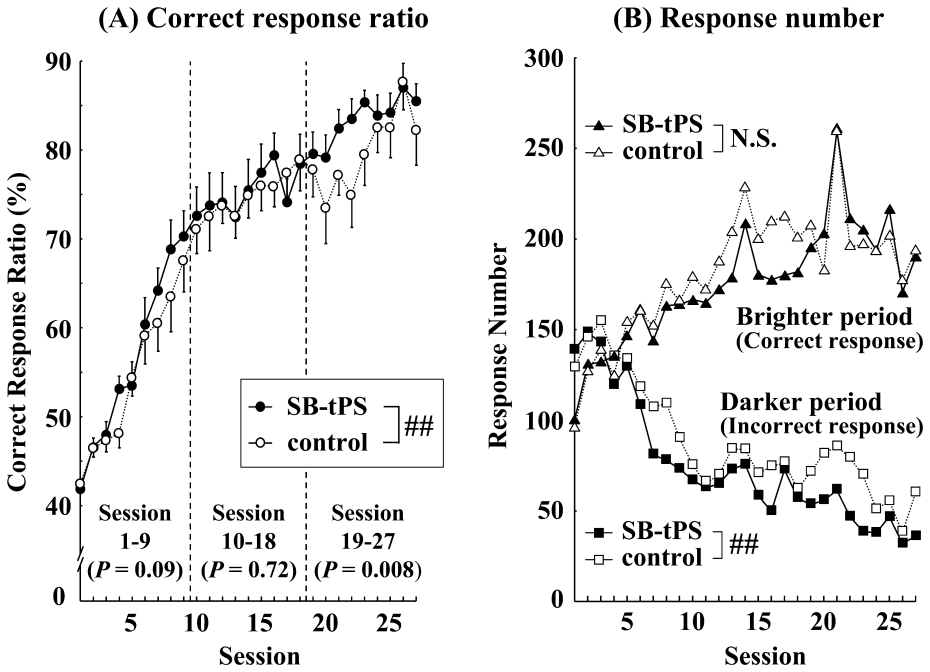

Fig. 3. Effect of oral administration of SB-tPS (50 mg/kg) on the brightness discrimination test. A: Correct response ratio in each session. Data represent the mean ± S.E.M. (n = 10). *P* values of each 3 terms of 9 sessions (2-way ANOVA) are also shown. B: Response number of each session in both brighter and darker period. Data represent mean values (n = 10). Comparisons between two groups in the total sessions (session 1 to 27) were carried out by 2-way ANOVA. $^{\#\#}P<0.01$. N.S. = not significant.

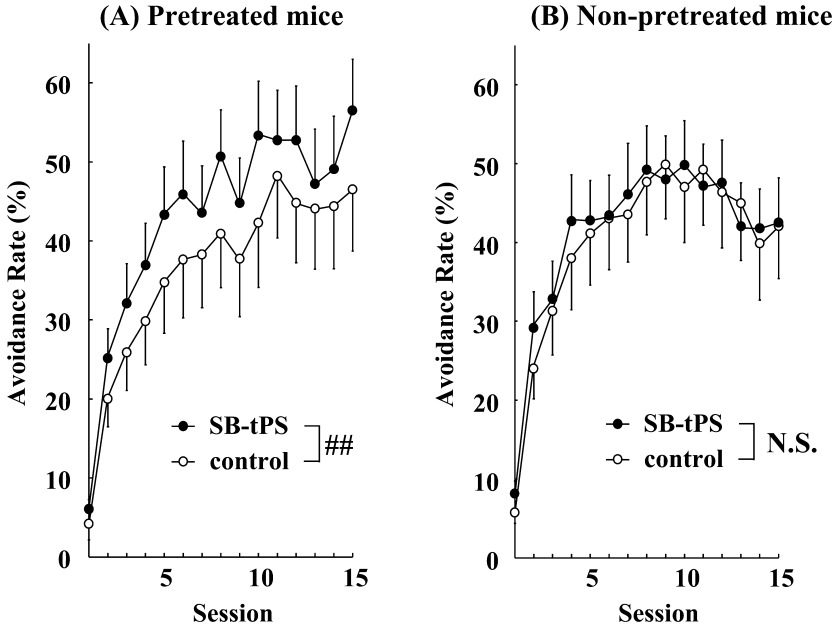

Fig. 4. Effect of SB-tPS pretreatment on the active avoidance test. A: SB-tPS administration (50 mg/kg, p.o.) was started 4 weeks before the test. B: SB-tPS was administrated only during the test period. Data represent the mean ± S.E.M. (n = 20). Comparisons between two groups in the total sessions (session 1 to 15) were carried out by 2-way ANOVA. $^{\#\#}P<0.01$. N.S. = not significant.

difference was detected in each session (Wilcoxon rank-sum test).

Discussion

In this study, we evaluated the nootropic effects of SB-tPS in several learning and memory tests and found that repeated oral administration of SB-tPS raised the active avoidance rate (Fig. 2A) and the correct response ratio in brightness discrimination (Fig. 3A).

It has been confirmed that SB-tPS does not alter the pain threshold of mice in the hot plate test (9). In addition, as mentioned above, SB-tPS did not affect any parameters in the open-field test (Table 1). The improved performance in Fig. 2 is therefore not derived from changes in general or emotional behavior.

In the active avoidance test, about one third of the mice in both groups seldom avoided the electrical shock in the early session (sessions 1 to 7). One explanation is that they spent most of the test period in a freezing or crouching posture without touching the lever. These mice did not seem to acquire the normal avoidance response, and we therefore defined mice whose avoidance rate was below 10% in every early session from 1 to 7 as the low responding (LR) group and the rest of the mice as the high responding (HR) group.

In the HR group, mice treated with SB-tPS showed a significantly higher avoidance rate than control mice (Fig. 2B), clearly showing that SB-tPS could enhance the learning ability in normal adult animals. In the LR group, the control mice could not avoid the electrical shock even in the last session, but 3 out of 7 mice treated with SB-tPS developed avoidance in the later session (Fig. 2B). This result is consistent with the report that an intraperitoneal administration of BC-PS (15 mg/kg for 30 days) increased the avoidance performance of low-responding rats (18) and may be partly explained by the anti-depressive effect of PS (22).

In addition to the active avoidance test, the brightness discrimination test was used to evaluate learning ability. Contrary to the active avoidance test, the method of the brightness discrimination test includes shaping stage in which rats are trained to press the lever for food pellets prior to the discrimination stage. Therefore, individual differences in response to the lever shown in the active avoidance test could be excluded. The improvement of the correct response ratio by SB-tPS (Fig. 3A) also supported the hypothesis that SB-tPS could enhance the learning ability of normal adult animals. As shown in Fig. 3B, SB-tPS decreased the incorrect responses (responses to dark stimuli), and the increase in the correct response ratio was therefore not due to increased random lever pressing during the test. A similar observation was noted in the active avoidance test in Experiment 1. Mice treated with SB-tPS pressed the lever less often than control mice during ITI in the active avoidance test (data not shown).

The brightness discrimination test was started without SB-tPS pretreatment to exclude its influence on the acquisition of lever-pressing performance (shaping stage). This may be one reason why the difference in the correct response ratio in this test was not clear compared with the active avoidance test in which SB-tPS was pretreated. When the test period was divided into three (the first 9, middle 9, and last 9 sessions), the difference in the correct response ratio only appeared in the last 9 sessions (Fig. 3A).

To elucidate the necessity of SB-tPS pretreatment, another active avoidance test was carried out and the performance of mice that had been given SB-tPS for 4 weeks before the test was compared with that of mice that received SB-tPS only during the 15-day test period. Since the effect of PS was observed within 27 days in experiment 2, we considered that 4 weeks was enough to evaluate the necessity of pretreatment. As shown in Fig. 4A, the 4-week pretreated mice showed a significant increase in avoidance rate, while mice treated with SB-tPS only during the test period did not (Fig. 4B). These results (Experiments 2 and 3) suggest that enhanced learning ability by SB-tPS may be achieved after 4 weeks from the first treatment. In this study, however, the SB-tPS administration conditions were fixed to 50 mg/kg per day for about one month, and it therefore remains unclear whether one-month pretreatment of SB-tPS is necessary to improve the learning ability of normal adult rodents.

Since PS is an important constituent of neuronal membranes, its effect on the membranous environment is proposed as a mechanism of SB-tPS. PS increased the fluidity of dog synaptosomal plasma membranes and Na^+,K^+-ATPase activity (23), and in our previous study, the improvement of synaptosomal Na^+,K^+-ATPase activity of aged rats by the oral administration of SB-tPS was observed (11). PS is also known to affect the exocytosis of neurotransmitters by interacting with membrane-binding proteins (24 – 27), and therefore it is possible that repeated administration of SB-tPS can induce membranous changes in neurons and thereby enhance the learning activity of normal adult rodents.

Actually, there are many reports showing the effect of PS on cholinergic neurotransmission. We have already reported that acetylcholine (ACh) release from the cerebral cortical synaptosomes of young mice was increased by the in vitro treatment of SB-tPS (28) and that the oral administration of SB-tPS (60 mg/kg for 60 days) to aged rats improved not only the performance

in the Morris water maze task but also in synaptosomal ACh release (11). As enhanced ACh release during the acquisition of lever-pressing operant behavior was observed in microdialysis studies (29, 30), the action of SB-tPS on learning ability could be partly explained by changes in these cholinergic functions.

Recently, transgenic mice overexpressing the NMDA receptor 2B in the forebrain (31) or growth-associated protein-43 (GAP-43) (32) were reported to show enhanced learning and memory activity. Interestingly, Cohen et al. (33) reported that the repeated intraperitoneal administration of PS (20 mg/kg per day) for 3 weeks restored the age-related decrease in NMDA receptor density in the forebrain of mice. In addition, Gianotti et al. (34) reported that the 17-day intraperitoneal administration of PS (15 mg/kg per day) improved the age-induced deterioration of endogenous GAP-43 phosphorylation, although 7 days' administration was insufficient.

These observations are well consistent with the results of this study in which about one-month pretreatment of SB-tPS was necessary to enhance the learning ability of normal adult rodents (Fig. 4). The NMDA receptor and GAP-43 could be considered key components of long-term potentiation (LTP), which is accepted as the molecular basis of memory and learning (35). We are interested in the possibility of whether the same action of PS could occur in the brain of normal adult rodents as aged rodents.

In conclusion, this study demonstrated that orally administered SB-tPS enhances the learning ability of normal adult rodents, suggesting that SB-tPS may be useful for complementary and alternative medicine as well as cognitive disease.

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