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Theobromine upregulates cerebral brain-derived neurotrophic factor and facilitates motor learning in mice

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44 Keywords:

45 cAMP, phosphodiesterase, motor learning, VASP phosphorylation, BDNF, CREB

46 phosphorylation

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Abstract

Theobromine, which is a caffeine derivative, is the primary methylxanthine produced by *Theobroma cacao*. Theobromine works as a phosphodiesterase (PDE) inhibitor to increase intracellular cyclic adenosine monophosphate (cAMP). cAMP activates the cAMP-response element-binding protein (CREB), which is involved in a large variety of brain processes, including the induction of the brain-derived neurotrophic factor (BDNF). BDNF supports cell survival and neuronal functions, including learning and memory. Thus, cAMP/CREB/BDNF pathways play an important role in learning and memory. Here, we investigated whether orally administered theobromine could act as a PDE inhibitor centrally and affect cAMP/CREB/BDNF pathways and learning behavior in mice.

The mice were divided into two groups. The control group (CN) was fed a normal diet, whereas the theobromine group (TB) was fed a diet supplemented with 0.05% theobromine for 30 days. We measured the levels of theobromine, phosphorylated vasodilator-stimulated phosphoprotein (p-VASP), phosphorylated CREB (p-CREB), and BDNF in the brain. p-VASP was used as an index of cAMP increases. Moreover, we analyzed the performance of the mice on a three-lever motor learning task.

Theobromine was detectable in the brains of TB mice. The brain levels of p-VASP, p-CREB, and BDNF were higher in the TB mice compared with those in the CN mice. In addition, the TB mice performed better on the three-lever task than the CN mice did. These results strongly suggested that orally administered theobromine acted as a PDE inhibitor in the brain, and it augmented the cAMP/CREB/BDNF pathways and motor learning in mice.

Keywords:

cAMP, phosphodiesterase, motor learning, VASP phosphorylation, BDNF, CREB phosphorylation

1. Introduction

In South America, chocolate has a long history dating back to at least 600 B.C. [1]. However, the first Westerner to eat chocolate is thought to be the 16th century Spanish general, Hernando Cortes [2]. Recently, consumption of chocolate with a high concentration of cacao (*Theobroma cacao*) has become popular around the world because cacao contains many flavonoids that have pleiotropic roles in neuroprotection and cognition [3, 4]. In addition, cacao contains theobromine, which is a caffeine derivative [5]. Theobromine is the primary methylxanthine found in products of *Theobroma cacao* [6]. Thus, an increase in chocolate intake results in an increase in the uptake of theobromine as well as flavonoids.

Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger that transduces extracellular signals into intracellular responses by communicating with downstream targets in the cascade [7]. cAMP signaling is fundamentally involved in neural wiring and the brain mechanisms that mediate cognitive processes [8-10].

Intracellular cAMP is synthesized from adenosine triphosphate by adenylyl cyclases and hydrolyzed by cyclic nucleotide phosphodiesterases (PDEs). cAMP activates the cAMP-response element-binding protein (CREB) through protein kinase A (PKA) activation [8-10]. The activation of CREB, which is a nuclear transcription factor, is

involved in a large variety of brain processes, including the induction of brain-derived neurotrophic factor (BDNF) [11]. BDNF supports cell survival and neuronal functions, including the neuroplasticity that mediates learning and memory [11]. Thus, the cAMP/CREB/BDNF pathways play important roles in cognitive processes, including learning and memory. Consequently, there has been significant interest in targeting PDEs as cognition-enhancing drugs [12-14].

Methylxanthines, including theobromine, are well-known inhibitors of PDEs [15-18]. We have previously confirmed that theobromine increases the intracellular cAMP concentration in glioblastomas [19]. Thus, theobromine might enhance cognitive function through cAMP/CREB/BDNF pathways. However, the *in vivo* physiological and pharmacological actions of theobromine have not been fully elucidated. In this study, we examined whether orally administered theobromine enhanced the cAMP/CREB/BDNF pathways and/or cognitive function by conducting the following experiments in theobromine-fed mice. First, we measured the theobromine concentrations in the plasma and cerebral cortex. Second, we analyzed the levels of the vasodilator-stimulated phosphoprotein (VASP) in the cerebral cortex. VASP is an established substrate for PKA, and its phosphorylation reflects PKA activation following an increase in cAMP [20-22]. Third, we analyzed the activation of CREB in

the cerebral cortex and hippocampus. Fourth, we measured the BDNF levels in the cerebral cortex and hippocampus. Finally, we analyzed the performance of the mice on a three-lever operant task that was developed to study several different aspects of motor learning, including sequence learning, skill learning, adaptation, and reversal learning, in mice [23]. We found that the theobromine-fed mice performed better on the three-lever motor learning task. In addition, the cAMP/CREB/BDNF pathways were enhanced in these mice.

2. Materials and Methods

2.1 Animals

Male C57BL/6NCr mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and housed in a room with controlled temperature ($23 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 10\%$) and under a 12/12 h light–dark cycle. They had access to food and water *ad libitum*. At the end of the experiments, the mice were sacrificed with an overdose of isoflurane. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Shimane University Faculty of Medicine and the animal welfare committee of Kanazawa University, which complied with the Guidelines for Animal

Experimentation of the Japanese Association for Laboratory Animal Science.

2.2 Materials

Theobromine, caffeine-*d*₉, and the Glucose CII-Test WAKO Kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The BDNF Emax® ImmunoAssay System was purchased from Promega Corporation (Madison, WI, USA). Anti-phospho-VASP (Ser239), anti-βactin, anti-CREB, anti-phospho-CREB (Ser133), horseradish peroxidase (HRP)-linked anti-mouse IgG, and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.3 Feeding and experiment schedules

The feeding and experiment schedules are summarized in Figure 1. All of the mice had free access to a standard chow (CRF-1, Oriental Yeast Co., LTD., Tokyo, Japan) for several days after admission. On Day0, the mice were divided into two groups. The first group, which consisted of the control (CN) mice, was fed the CRF-1 chow for 30 days and then subsequently switched to the CE-2 chow (O'HARA & Co., Ltd., Tokyo, Japan) for the remaining 30 days. The second group of mice (TB) was fed the standard CRF-1 chow that was supplemented with 0.05% (W/W) of theobromine (Oriental Yeast Co.,

LTD.) for 30 days and then switched to the CE-2 chow for the remaining 30 days.

During the period of operant task performance (Day30 to Day60), the CE-2 chow (50 mg/1 pellet) was used instead of the CRF-1 chow (3–4 g/1 pellet) to adjust for the total amount of food per day (1.5–2.5 g).

The concentration of theobromine (0.05%) was selected according to the caffeine-supplemented chow [24] because theobromine is a caffeine derivative. The length of time for treatment (30 days) was selected based on our preliminary data with rats that showed the theobromine concentration in the plasma gradually increased and reached submaximal levels 30 days after switching to the theobromine-supplemented chow (unpublished preliminary data).

The body weights of the mice were measured at the beginning (Day0; start of theobromine feeding), halfway point (Day30; switching to CE-2 chow), and end (Day60; finish) of the experiments. The theobromine concentrations in the plasma and cerebral cortex were measured at the halfway point (Day30) and on the final (Day60) day of the experiments. The levels of plasma glucose, phosphorylated VASP, CREB, and BDNF were measured at the halfway (Day30) point of the experiments. The lever operant tasks were performed for a 30-d period from the halfway point (Day30) until the final day (Day60) of the experiments.

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173 **2.4 Measurements of plasma glucose**

174 The plasma glucose concentrations were determined with a Glucose CII-Test WAKO
175 Kit (Wako Pure Chemical Industries, Ltd.) according to the kit's instructions.

176

177 **2.5 Measurements of theobromine content**

178 Theobromine was measured in the biological samples as previously described, with a
179 slight modification [25]. The brain tissues were homogenized in four volumes (w/v) of
180 phosphate-buffered saline (pH 7.4). We mixed 50 μ L of plasma and brain homogenate
181 with 250 ng of caffeine-*d*9 in 200 μ L of acetonitrile and then kept the samples at -30°C
182 for 30 min. The samples were centrifuged at $5,000 \times g$ for 10 min at 4°C to remove the
183 precipitated proteins. The supernatants were analyzed with high-performance liquid
184 chromatography in combination with electrospray ionization-mass spectrometry that
185 was performed with a TSQ quantum mass spectrometer (Thermo Fisher Scientific K.K.,
186 Tokyo, Japan). The high-performance liquid chromatography was performed with a
187 Luna 3- μ m C18(2) 100Å liquid chromatography column (100 $\times$ 2.0 mm, Phenomenex,
188 Inc., Torrance, CA, USA) at 30°C. The samples were eluted in a mobile phase
189 consisting of acetonitrile–methanol (4:1, v/v) and water–acetic acid (100:0.1, v/v) in a

10:90 ratio for 2 min. After 5 min, the ratio was changed to 70:30 and maintained for 7 min. Subsequently, the ratio was changed to 80:20 and held for 2 min. Finally, after 9 min, the ratio was changed to 100:0 and held for 2 min with a flow rate of 0.1 mL/min. Tandem mass spectrometry analyses were conducted in the positive ion mode, and theobromine (m/z 180.8>163.1) and caffeine- d_9 (m/z 204.2>144) were detected and quantified with selected reaction monitoring. The peaks were selected, and their areas were calculated with Xcalibur™ 2.1 software (Thermo Fisher Scientific K.K.).

2.6 Levels of BDNF, phosphorylated VASP, and CREB

The cerebral cortex and hippocampus were homogenized in radioimmunoprecipitation assay buffer with a glass homogenizer. Subsequently, the samples were centrifuged at $800 \times g$ for 15 min at 4°C to remove tissue debris, and a protein assay was performed to determine the protein concentrations. Equal amounts of protein were analyzed with the BDNF Emax® ImmunoAssay System (Promega Corporation) according to the kit's instructions. Western blotting was performed as previously described [26]. The proteins in the cerebral cortex and hippocampus were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred onto polyvinylidene fluoride membranes and incubated with primary antibodies (1:1,000)

and then HRP-linked secondary antibodies (1:2,000). The blots were developed with the Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore Corporation, Billerica, MA, USA).

2.7 One-lever and three-lever operant tasks

The one-lever and three-lever operant tasks were performed as previously described [23]. Ten CN mice and 10 TB mice that were 6 weeks old on Day0 were kept separately in plastic cages with four compartments (KN-606, 230 × 300 × 130 mm, Natsume Seisakusho Co., Ltd., Tokyo, Japan) and provided a limited amount of food. Before the training, the mice were handled for approximately 10 min/day for one week in order for the mice to habituate to the testing area and experimenter. One 60-min training session was conducted once a day five days a week (Monday to Friday). The experiments were performed in an operant chamber (225 × 240 × 200 mm, OP-3101K, O'HARA & Co., Ltd.) that was placed in a sound-attenuating box (495 × 750 × 685 mm). Three levers (18 × 15 mm) protruded into the chamber, and the right (A), center (B), and left (C) levers were positioned 2, 4, and 2 cm, respectively, above the floor. The B-lever was set 2 cm higher than the other two levers so that the mouse pressed the B-lever with a forelimb by standing up on its hind legs. The Operant Task for multi levers program

226 (O'HARA & Co., Ltd.) controlled the execution of all experiments and data collection.
227 When the mouse pressed an active lever (one-lever task) or three levers in the correct
228 order within a given time (three-lever task), one pellet (AIN-76A, 10 mg, Research
229 Diets, Inc., New Brunswick, NJ, USA) was delivered from the automatic diet feeder for
230 reinforcement (PD-010D, O'HARA & Co., Ltd). The required load for the lever press
231 was adjusted to 4–7 g. The number of reinforcements (R, pellets) and presses on each
232 lever were recorded on a personal computer through an interface unit (AOI040C,
233 O'HARA & Co., Ltd.) by the task program. In the operant chamber, water was available
234 *ad libitum*.

235 The one-lever task was used as a shaping procedure for the three-lever task. In
236 this task, the mouse was trained to press any one of the active levers for a food reward
237 (fixed ratio 1, FR1). The number of active levers was initially set to three. When the
238 mouse pressed the same active lever more than 100 times per session in two consecutive
239 sessions, the lever was inactivated in the subsequent sessions. The one-lever task was
240 completed when the mouse pressed the last active lever more than 100 times per session
241 in two consecutive sessions.

242 The mouse was then trained to press the three levers in an ABC sequence
243 (A-lever, B-lever, and C-lever). In this three-lever task, the mouse was required to press

the second (or third) lever within a given time (T) after the onset of the first (or second) lever press. Initially, T was set to 99.9 s, and it was sequentially decreased to 3 s, 2 s, and 1 s when R was over 100 in two consecutive sessions. After completion of the three-lever task, the mouse was trained to press the three levers in the opposite sequence (CBA; C-lever, B-lever, and A-lever). Like the original three-lever task, T was initially set to 99.9 s, and it was sequentially decreased to 3 s, 2 s, and 1 s when R was over 100 in two consecutive sessions.

2.8 Statistical analysis

The data are expressed as mean $\pm$ standard error of the mean. Statistical significance was evaluated with a two-way analysis of variance (ANOVA) or Student's *t*-test. *P* values less than 0.05 were considered statistically significant. The *p* value, effect size (*d*), and power (1- β) were obtained with Statcel software.

3. Results

3.1 Body weights and plasma glucose levels

First, we measured the body weights and plasma glucose levels. The body weights of

the mice fed the theobromine-supplemented chow (TB mice, n = 10) did not differ from those of the mice fed the standard chow (CN mice, n = 10) on Day0 ($p = 0.15$), Day30 ($p = 0.59$), or Day60 ($p = 0.57$) (Table 1). The plasma glucose levels of the TB mice (n = 6) did not differ from those of the CN mice (n = 6) on Day30 ($p = 0.52$; Table 2). These results indicated that theobromine did not affect the feeding behavior or glucose metabolism of the mice.

3.2 Theobromine concentrations in the plasma and brain

Next, we examined whether theobromine was taken up into the brain. In the CN mice (n = 6), theobromine was not detected in the plasma or cerebral cortex on Day30 or Day60 (Table 2). In the TB mice (n = 6), the theobromine concentrations in the plasma and cerebral cortex were 2.20 ± 0.12 $\mu\text{g/mL}$ and 0.21 ± 0.02 $\mu\text{g/mL}$, respectively, on Day30 (Table 2). However, theobromine was no longer detected in the plasma and cerebral cortex 30 days after the theobromine-supplemented chow was replaced with the standard chow (Day 60; Table 2). The presence of theobromine in the brains of the TB mice indicated that orally administered theobromine influenced the signaling pathways in the brain through its pharmacological actions.

3.3 Levels of phosphorylated VASP, phosphorylated CREB, and BDNF in the brain

Next, we examined whether orally administered theobromine enhanced the cAMP/CREB/BDNF pathway in the brain in the CN mice ($n = 6$) and TB mice ($n = 6$) on Day30. The levels of phosphorylated VASP protein in the cerebral cortex were significantly higher in the TB mice than in the CN mice ($p < 0.05$) (Fig. 2), which indicated that the TB mice had increased levels of intracerebral cAMP. The levels of phosphorylated CREB in both the hippocampus and cerebral cortex were significantly higher in the TB mice than in the CN mice ($p < 0.05$; Fig. 3). The protein expression levels of BDNF in the hippocampus and cerebral cortex were also significantly higher in the TB mice than in the CN mice ($p < 0.01$ and $p < 0.05$, respectively; Table 2). These results indicated that orally administered theobromine activated the cAMP/CREB/BDNF pathways in the brain.

3.4 One-lever and three-lever operant tasks

Finally, we analyzed the performances of the CN mice ($n = 10$) and TB mice ($n = 10$) on the three-lever operant task. The CN mice and TB mice generally exhibited similar performances on the one-lever task (Fig. 4), which was used as a shaping procedure for the three-lever task. The total number of sessions required to complete the one-lever

task was 10.3 ± 0.7 sessions in the CN mice and 10.3 ± 0.3 sessions in the TB mice (Fig. 4B), which was not a significant difference ($p = 0.99$). In the first several sessions, both types of mice exhibited similar increases in the total number of lever presses (Fig. 4A). A two-way ANOVA (session $\times$ mouse group) showed no significant interaction effects of session and mouse group ($p = 0.88$) and a significant main effect of session ($p < 0.001$) but not mouse group ($p = 0.23$).

In the three-lever task, the TB mice exhibited a significantly better performance than the CN mice did (Fig. 5). In the first several sessions, the total number of lever presses (Fig. 5A) exhibited a significant interaction effect of session and mouse group ($p < 0.01$). In addition, the total number of presses was significantly higher in the TB mice than in the CN mice in the first session ($p < 0.05$) but not in the subsequent sessions ($p > 0.05$). The number of reinforcements, success rates, and number of lever presses for the ABCABC pattern were significantly increased in the TB mice than in the control mice (Fig. 5B-D). A two-way ANOVA showed no significant interaction effects of session and mouse group (reinforcement, $p = 0.35$; success rate, $p = 0.83$; ABCABC, $p = 0.09$) and significant main effects of session (reinforcement, success rate, and ABCABC, $p < 0.001$) and mouse group (reinforcement, $p < 0.01$, $d = 0.28$, $1 - \beta = 0.79$; success rate, $p < 0.001$, $d = 0.45$, $1 - \beta = 0.99$; ABCABC, $p < 0.01$, $d = 0.35$, $1 - \beta =$

0.94). The total number of sessions required to complete the three-lever task differed significantly between the CN and TB mice (CN mice, 9.6 ± 0.4 sessions; TB mice, 8.3 ± 0.1 sessions; $p < 0.05$). These results demonstrated that the TB mice performed significantly better on the three-lever operant task compared with the CN mice.

Better performances of the TB mice were also observed on the reverse three-lever task (CBACBA), although the differences in the performances between the two groups were less obvious. The total numbers of sessions required to complete the reverse three-lever task were similar (CN mice, 8.4 ± 0.2 sessions; TB mice, 8.3 ± 0.2 sessions; $p = 0.45$). The total number of lever presses (Fig. 6A) exhibited no significant interaction effects of session and mouse group ($p = 0.59$) and significant main effects of both session ($p < 0.001$) and mouse group ($p < 0.05$, $d = 0.23$, $1 - \beta = 0.63$). The number of reinforcements and success rates showed no significant interaction effects of session and mouse group (reinforcement, $p = 0.79$; success rate, $p = 0.83$) and a significant main effect of session (reinforcement and success rate, $p < 0.001$) but not mouse group (reinforcement, $p = 0.75$, $d = 0.03$, $1 - \beta = 0.06$; success rate, $p = 0.06$, $d = 0.20$, $1 - \beta = 0.51$). Importantly, the number of lever presses for the CBACBA pattern (Fig. 6D) was significantly increased in the TB mice compared with the CN mice. Session and mouse group did not exhibit a significant interaction effect ($p = 0.70$), while they did show

significant main effects (session, $p < 0.001$; mouse group, $p < 0.05$, $d = 0.26$, $1 - \beta = 0.73$).

4. Discussion

Several lines of evidence have shown that theobromine exerts a variety of physiological and pharmacological actions [6, 27, 28]. Notably, theobromine functions as an inhibitor of PDEs, including PDE4 [19], which results in an increase in intracellular cAMP. PDE4 inhibitors prevent cognitive deficits. Therefore, theobromine might serve as a potential protective agent against cognitive disorders [13, 14]. This possibility was supported by the results of the present study, which demonstrated that orally administered theobromine influenced signaling pathways in the brain, including those for cAMP, CREB, and BDNF, and facilitated motor learning in mice.

In order to exert its effect against cognitive disorders, theobromine must be taken up into the brain. Our data showed that theobromine was detectable in the plasma and cerebral cortex of the mice 30 days after initiation of the theobromine-supplemented chow. The plasma and brain levels of theobromine were about 2.2 and 0.2 $\mu\text{g/mL}$, respectively. The level of 0.2 $\mu\text{g/mL}$ is sufficient to produce pharmacological effects

[29]. However, the concentrations differed greatly between the plasma and brain. The molecular structure of theobromine is very similar to that of caffeine. Caffeine freely crosses the blood-brain barrier (BBB), and its concentration in the brain is almost the same as it is in plasma [30-32]. Thus, the permeability of the BBB to theobromine would be much lower than that to caffeine.

Isoflurane, which was used as an anesthetic agent in the present study, is thought to have effects on the permeability of the BBB [33, 34]. Therefore, we cannot exclude the possibility that our biochemical data might have been affected by alterations in the BBB that were induced by isoflurane. The BBB selectively controls the homeostasis of the central nervous system environment through specific structural and biochemical features of endothelial cells, pericytes, and glial cells [35]. In addition, tight junction molecules between the brain endothelial cells contain the molecular components of the BBB [33]. Alterations to the cellular and molecular structures may result in BBB disruption. Isoflurane inhibits the expression of occludin, which is a tight junction molecule, and influences BBB integrity [34]. However, the downregulation of occludin takes time, and a 1-h exposure to isoflurane *in vivo* does not alter the morphology of the BBB visualized with electron microscopy [36]. In the present study, the exposure to isoflurane was only a few minutes. Thus, the BBBs of the mice used in

the present study were assumed to be relatively intact.

The present study demonstrated that orally administered theobromine enhanced the cAMP/CREB/BDNF pathways in the brain and motor learning. Theobromine, which is a well-known inhibitor of PDEs [19], was expected to increase the concentration of cAMP by preventing the hydrolysis of cAMP. We showed that the levels of phosphorylated VASP protein in the cerebral cortex were significantly higher in the TB mice compared with the CN mice (Fig. 2). Because VASP is an established substrate of PKA, increased levels of phosphorylated VASP indicated increased cAMP and the resulting PKA activation [20-22]. Therefore, our data strongly suggested that orally administered theobromine acted as a PDE inhibitor and increased the cAMP levels in the brain. cAMP plays an important role in various neural functions [7, 37, 38] as a second messenger molecule. In particular, cAMP signaling is involved in synaptic plasticity, such as long-term potentiation [8, 9, 39-41], which is crucial for learning and memory [8, 9]. Furthermore, cAMP is known to activate CREB through PKA activation [8, 9]. In addition, CREB has been implicated in long-term potentiation and the production of BDNF. BDNF is one of several neurotrophins that regulate synaptic plasticity [8, 9, 39-41]. Thus, the cAMP/CREB/BDNF pathways are heavily involved in learning and memory. In the present study, we observed that the levels of

phosphorylated CREB and BDNF, as well as phosphorylated VASP, were increased in the brains of TB mice when they were measured 30 days after the start of the theobromine-supplemented chow (Table 2 and Fig. 3). These results indicated that orally administered theobromine facilitated the cAMP/CREB/BDNF pathways in the brain. More importantly, we observed that orally administered theobromine enhanced motor learning (Figs. 5 and 6). Our observations were in agreement with previous findings concerning the roles of the cAMP/CREB/BDNF pathways in learning and memory.

Lastly, the question remains as to how long the effects of theobromine on the brain signaling pathways and functions last. Theobromine was not detectable in the plasma and brains of the mice in the TB group 30 days after replacement (Day60) of the theobromine-supplemented chow with the standard chow. However, the theobromine-mediated enhancement of motor learning was observed between Day30 and Day60. When the theobromine disappears in the plasma and brain after the theobromine chow is stopped is unclear.

5. Conclusions

This study demonstrated that orally administered theobromine acted as a PDE inhibitor in the brain, influenced the signaling pathways, including those for cAMP, CREB and BDNF, and facilitated motor learning in mice. We confirmed that theobromine was present in the brains of the theobromine-fed mice and that it increased the cAMP levels. cAMP signaling mediates numerous neural processes, including development, cellular excitability, synaptic plasticity, learning, and memory. Increased cAMP phosphorylates CREB and, in turn, releases BDNF. The cAMP, CREB, and BDNF pathways mediate synaptic plasticity, thus facilitating behavioral learning and memory. Therefore, our findings strongly suggested that cacao products, including chocolate and cocoa, can benefit learning and memory.

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Conflicts of interest disclosure

424 The authors do not have any conflicts of interest to declare.

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Table 1

Body Weights [g, mean $\pm$ standard error of the mean (SEM)] in control (CN) and theobromine-fed (TB) mice

	CN (n = 10)	TB (n = 10)	<i>P</i> value
Initial			
(Day0)	21.1 $\pm$ 0.4	21.9 $\pm$ 0.3	<i>P</i> = 0.15
Halfway			
(Day30)	24.6 $\pm$ 0.4	24.3 $\pm$ 0.4	<i>P</i> = 0.59
Final			
(Day60)	24.9 $\pm$ 0.5	24.5 $\pm$ 0.3	<i>P</i> = 0.57

Table 2

Glucose levels in plasma, theobromine levels in plasma and the cerebral cortex, and brain-derived neurotrophic factor (BDNF) levels in the hippocampus and cerebral cortex in control (CN) and theobromine-fed (TB) mice

	CN (n = 6)	TB (n = 6)	<i>P</i> value
Glucose (Day30)			
(mg/dL plasma)	254.6 ± 13.85	241.35 ± 12.91	<i>P</i> = 0.52
Theobromine (Day30)			
(µg/mL plasma)	Not detected	2.20 ± 0.12	
(µg/mL cerebral cortex)	Not detected	0.21 ± 0.02	
Theobromine (Day60)			
(µg/mL plasma)	Not detected	Not detected	

601	($\mu\text{g/mL}$ cerebral cortex)	Not detected	Not detected	
602				
603				
604	BDNF (Day30)			
605	(pg/g hippocampus)	25.81 ± 0.85	29.60 ± 0.99	$P = 0.0086$
606	(pg/g cerebral cortex)	20.28 ± 0.61	22.67 ± 0.82	$P = 0.029$
607				
608				
609	Each value represents the mean $\pm$ SEM			
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Figure legends

Figure 1

Feeding and experiment schedules. The mice were divided into two groups. The mice in the first group (control; CN) were fed the standard chow (CRF-1) for 30 days and were then switched to CE-2 for 30 days. The second group (theobromine; TB) was fed chow supplemented with 0.05% (W/W) theobromine (CRF-1 with 0.05% of theobromine) for 30 days and were then switched to CE-2 for 30 days. Body weight was measured on Day0, Day30, and Day60. The theobromine concentrations in plasma and the cerebral cortex were measured on Day30 and Day60. The plasma glucose, brain-derived neurotrophic factor (BDNF), phosphorylated vasodilator-stimulated phosphoprotein (VASP), and cAMP-response element-binding protein (CREB) levels were measured on Day30. The Lever operant tasks were performed between Day30 and Day60.

Figure 2

Phosphorylated VASP levels in the cerebral cortex of the mice. Theobromine induces VASP phosphorylation in the cerebral cortex of mice. Each data point represents the mean $\pm$ SEM (n = 6, * P < 0.05)

Figure 3

Phosphorylated CREB levels in the hippocampus and cerebral cortex of mice. Theobromine induces CREB phosphorylation in the hippocampus and cerebral cortex of the mice. Each data point represents the mean $\pm$ SEM ($n = 6$, $*P < 0.05$).

Figure 4

Performance on the one-lever task. The time course of the total number of lever presses during the 1st–8th sessions (A) and the number of sessions required for the completion of the one-lever task (B). Each data point represents the mean $\pm$ SEM ($n = 10$). Statistical significance was evaluated with a two-way analysis of variance (ANOVA; A) or Student's t -test (B).

Figure 5

Performance on the three-lever task. The time courses of the total number of lever presses (A), number of reinforcements (B), success rates (C), and number of lever presses for the A→B→C→A→B→C (ABCABC) pattern (D) during the 1st–5th sessions. Each data point represents the mean $\pm$ SEM ($n = 10$). Statistical significance was evaluated with a two-way ANOVA. Differences with P values less than 0.05 were

considered significant.

Figure 6

Performance on the reverse three-lever task. The time courses of the total number of lever presses (A), number of reinforcements (B), success rates (C), and number of lever presses for the C→B→A→C→B→A (CBACBA) pattern (D) during the 1st–5th sessions. Each data point represents the mean ± SEM (n = 10). Statistical significance was evaluated with a two-way ANOVA. Differences with *P* values less than 0.05 were considered significant.

Figure 1

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662

Cortex

Figure 2

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Figure 3

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1-lever

Figure 4

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3-lever

Figure 5

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Reverse 3-lever

Figure 6