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

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Keywords:

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

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

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

106 Methylxanthines, including theobromine, are well-known inhibitors of PDEs
107 [15-18]. We have previously confirmed that theobromine increases the intracellular
108 cAMP concentration in glioblastomas [19]. Thus, theobromine might enhance cognitive
109 function through cAMP/CREB/BDNF pathways. However, the *in vivo* physiological
110 and pharmacological actions of theobromine have not been fully elucidated. In this
111 study, we examined whether orally administered theobromine enhanced the
112 cAMP/CREB/BDNF pathways and/or cognitive function by conducting the following
113 experiments in theobromine-fed mice. First, we measured the theobromine
114 concentrations in the plasma and cerebral cortex. Second, we analyzed the levels of the
115 vasodilator-stimulated phosphoprotein (VASP) in the cerebral cortex. VASP is an
116 established substrate for PKA, and its phosphorylation reflects PKA activation
117 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 ± 2°C) and humidity (50 ± 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

136 Experimentation of the Japanese Association for Laboratory Animal Science.

137

138 **2.2 Materials**

139 Theobromine, caffeine-*d*9, and the Glucose CII-Test WAKO Kit were purchased from

140 Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The BDNF Emax® ImmunoAssay

141 System was purchased from Promega Corporation (Madison, WI, USA).

142 Anti-phospho-VASP (Ser239), anti-βactin, anti-CREB, anti-phospho-CREB (Ser133),

143 horseradish peroxidase (HRP)-linked anti-mouse IgG, and anti-rabbit IgG antibodies

144 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

145

146 **2.3 Feeding and experiment schedules**

147 The feeding and experiment schedules are summarized in Figure 1. All of the mice had

148 free access to a standard chow (CRF-1, Oriental Yeast Co., LTD., Tokyo, Japan) for

149 several days after admission. On Day0, the mice were divided into two groups. The first

150 group, which consisted of the control (CN) mice, was fed the CRF-1 chow for 30 days

151 and then subsequently switched to the CE-2 chow (O'HARA & Co., Ltd., Tokyo, Japan)

152 for the remaining 30 days. The second group of mice (TB) was fed the standard CRF-1

153 chow that was supplemented with 0.05% (W/W) of theobromine (Oriental Yeast Co.,

154 LTD.) for 30 days and then switched to the CE-2 chow for the remaining 30 days.
155 During the period of operant task performance (Day30 to Day60), the CE-2 chow (50
156 mg/1 pellet) was used instead of the CRF-1 chow (3–4 g/1 pellet) to adjust for the total
157 amount of food per day (1.5–2.5 g).

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

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

172

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 μL of plasma and brain homogenate

181 with 250 ng of caffeine-*d*9 in 200 μL of acetonitrile and then kept the samples at -30°C

182 for 30 min. The samples were centrifuged at 5,000 × *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-μm C18(2) 100Å liquid chromatography column (100 × 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 radioimmunopreciptation assay buffer with a glass homogenizer. Subsequently, the samples were centrifuged at 800 × *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)

208 and then HRP-linked secondary antibodies (1:2,000). The blots were developed with the
209 Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore Corporation,
210 Billerica, MA, USA).
211
212 **2.7 One-lever and three-lever operant tasks**
213 The one-lever and three-lever operant tasks were performed as previously described
214 [23]. Ten CN mice and 10 TB mice that were 6 weeks old on Day0 were kept separately
215 in plastic cages with four compartments (KN-606, 230 × 300 × 130 mm, Natsume
216 Seisakusho Co., Ltd., Tokyo, Japan) and provided a limited amount of food. Before the
217 training, the mice were handled for approximately 10 min/day for one week in order for
218 the mice to habituate to the testing area and experimenter. One 60-min training session
219 was conducted once a day five days a week (Monday to Friday). The experiments were
220 performed in an operant chamber (225 × 240 × 200 mm, OP-3101K, O'HARA & Co.,
221 Ltd.) that was placed in a sound-attenuating box (495 × 750 × 685 mm). Three levers
222 (18 × 15 mm) protruded into the chamber, and the right (A), center (B), and left (C)
223 levers were positioned 2, 4, and 2 cm, respectively, above the floor. The B-lever was set
224 2 cm higher than the other two levers so that the mouse pressed the B-lever with a
225 forelimb by standing up on its hind legs. The Operant Task for multi levers program

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

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

The mouse was then trained to press the three levers in an ABC sequence (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 ± 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 μg/mL and 0.21 ± 0.02 μg/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

298 task was 10.3 ± 0.7 sessions in the CN mice and 10.3 ± 0.3 sessions in the TB mice (Fig.
299 4B), which was not a significant difference ($p = 0.99$). In the first several sessions, both
300 types of mice exhibited similar increases in the total number of lever presses (Fig. 4A).
301 A two-way ANOVA (session × mouse group) showed no significant interaction effects
302 of session and mouse group ($p = 0.88$) and a significant main effect of session ($p <$
303 0.001) but not mouse group ($p = 0.23$).

304 In the three-lever task, the TB mice exhibited a significantly better performance
305 than the CN mice did (Fig. 5). In the first several sessions, the total number of lever
306 presses (Fig. 5A) exhibited a significant interaction effect of session and mouse group
307 ($p < 0.01$). In addition, the total number of presses was significantly higher in the TB
308 mice than in the CN mice in the first session ($p < 0.05$) but not in the subsequent
309 sessions ($p > 0.05$). The number of reinforcements, success rates, and number of lever
310 presses for the ABCABC pattern were significantly increased in the TB mice than in the
311 control mice (Fig. 5B-D). A two-way ANOVA showed no significant interaction effects
312 of session and mouse group (reinforcement, $p = 0.35$; success rate, $p = 0.83$; ABCABC,
313 $p = 0.09$) and significant main effects of session (reinforcement, success rate, and
314 ABCABC, $p < 0.001$) and mouse group (reinforcement, $p < 0.01$, $d = 0.28$, $1 - \beta = 0.79$;
315 success rate, $p < 0.001$, $d = 0.45$, $1 - \beta = 0.99$; ABCABC, $p < 0.01$, $d = 0.35$, $1 - \beta =$

316 0.94). The total number of sessions required to complete the three-lever task differed
317 significantly between the CN and TB mice (CN mice, 9.6 ± 0.4 sessions; TB mice, 8.3 ±
318 0.1 sessions; $p < 0.05$). These results demonstrated that the TB mice performed
319 significantly better on the three-lever operant task compared with the CN mice.
320 Better performances of the TB mice were also observed on the reverse
321 three-lever task (CBACBA), although the differences in the performances between the
322 two groups were less obvious. The total numbers of sessions required to complete the
323 reverse three-lever task were similar (CN mice, 8.4 ± 0.2 sessions; TB mice, 8.3 ± 0.2
324 sessions; $p = 0.45$). The total number of lever presses (Fig. 6A) exhibited no significant
325 interaction effects of session and mouse group ($p = 0.59$) and significant main effects of
326 both session ($p < 0.001$) and mouse group ($p < 0.05$, d = 0.23, 1 - β = 0.63). The number
327 of reinforcements and success rates showed no significant interaction effects of session
328 and mouse group (reinforcement, $p = 0.79$; success rate, $p = 0.83$) and a significant main
329 effect of session (reinforcement and success rate, $p < 0.001$) but not mouse group
330 (reinforcement, $p = 0.75$, d = 0.03, 1 - β = 0.06; success rate, $p = 0.06$, d = 0.20, 1 - β =
331 0.51). Importantly, the number of lever presses for the CBACBA pattern (Fig. 6D) was
332 significantly increased in the TB mice compared with the CN mice. Session and mouse
333 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 - β = 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 μg/mL, respectively. The level of 0.2 μg/mL is sufficient to produce pharmacological effects

352 [29]. However, the concentrations differed greatly between the plasma and brain. The
353 molecular structure of theobromine is very similar to that of caffeine. Caffeine freely
354 crosses the blood-brain barrier (BBB), and its concentration in the brain is almost the
355 same as it is in plasma [30-32]. Thus, the permeability of the BBB to theobromine
356 would be much lower than that to caffeine.
357 Isoflurane, which was used as an anesthetic agent in the present study, is
358 thought to have effects on the permeability of the BBB [33, 34]. Therefore, we cannot
359 exclude the possibility that our biochemical data might have been affected by alterations
360 in the BBB that were induced by isoflurane. The BBB selectively controls the
361 homeostasis of the central nervous system environment through specific structural and
362 biochemical features of endothelial cells, pericytes, and glial cells [35]. In addition, tight
363 junction molecules between the brain endothelial cells contain the molecular
364 components of the BBB [33]. Alterations to the cellular and molecular structures may
365 result in BBB disruption. Isoflurane inhibits the expression of occludin, which is a tight
366 junction molecule, and influences BBB integrity [34]. However, the downregulation of
367 occludin takes time, and a 1-h exposure to isoflurane *in vivo* does not alter the
368 morphology of the BBB visualized with electron microscopy [36]. In the present study,
369 the exposure to isoflurane was only a few minutes. Thus, the BBBs of the mice used in

370 the present study were assumed to be relatively intact.

371 The present study demonstrated that orally administered theobromine enhanced
372 the cAMP/CREB/BDNF pathways in the brain and motor learning. Theobromine, which
373 is a well-known inhibitor of PDEs [19], was expected to increase the concentration of
374 cAMP by preventing the hydrolysis of cAMP. We showed that the levels of
375 phosphorylated VASP protein in the cerebral cortex were significantly higher in the TB
376 mice compared with the CN mice (Fig. 2). Because VASP is an established substrate of
377 PKA, increased levels of phosphorylated VASP indicated increased cAMP and the
378 resulting PKA activation [20-22]. Therefore, our data strongly suggested that orally
379 administered theobromine acted as a PDE inhibitor and increased the cAMP levels in
380 the brain. cAMP plays an important role in various neural functions [7, 37, 38] as a
381 second messenger molecule. In particular, cAMP signaling is involved in synaptic
382 plasticity, such as long-term potentiation [8, 9, 39-41], which is crucial for learning and
383 memory [8, 9]. Furthermore, cAMP is known to activate CREB through PKA activation
384 [8, 9]. In addition, CREB has been implicated in long-term potentiation and the
385 production of BDNF. BDNF is one of several neurotrophins that regulate synaptic
386 plasticity [8, 9, 39-41]. Thus, the cAMP/CREB/BDNF pathways are heavily involved in
387 learning and memory. In the present study, we observed that the levels of

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

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

403

404

405 **5. Conclusions**

406 This study demonstrated that orally administered theobromine acted as a PDE inhibitor
407 in the brain, influenced the signaling pathways, including those for cAMP, CREB and
408 BDNF, and facilitated motor learning in mice. We confirmed that theobromine was
409 present in the brains of the theobromine-fed mice and that it increased the cAMP levels.
410 cAMP signaling mediates numerous neural processes, including development, cellular
411 excitability, synaptic plasticity, learning, and memory. Increased cAMP phosphorylates
412 CREB and, in turn, releases BDNF. The cAMP, CREB, and BDNF pathways mediate
413 synaptic plasticity, thus facilitating behavioral learning and memory. Therefore, our
414 findings strongly suggested that cacao products, including chocolate and cocoa, can
415 benefit learning and memory.
416
417
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422
423 **Conflicts of interest disclosure**

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

References

1. Hurst WJ, Tarka SM Jr, Powis TG, Valdez F Jr, Hester TR. Cacao usage by the earliest Maya civilization. Nature 2002; 418:289-90. doi:10.1038/418289a
2. Coe SD, Coe MD. The True History of Chocolate. London, Thames & Hudson; 1996.
3. Sokolov AN, Pavlova MA, Klosterhalfen S, Enck P. Chocolate and the brain: neurobiological impact of cocoa flavanols on cognition and behavior. Neurosci Biobehav Rev 2013; 37:2445-53. doi: 10.1016/j.neubiorev.2013.06.013.
4. Ramiro-Puig E, Castell M. Cocoa: antioxidant and immunomodulator. Br J Nutr 2009; 101:931-40. doi: 10.1017/S0007114508169896.
5. Pura Naik J. Improved high-performance liquid chromatography method to determine theobromine and caffeine in cocoa and cocoa products. J Agric Food Chem 2001; 49:3579-83.
6. Martínez-Pinilla E, Oñatibia-Astibia A, Franco R. The relevance of theobromine for the beneficial effects of cocoa consumption. Front Pharmacol 2015; 6:30. doi: 10.3389/fphar.2015.00030.
7. Francis SH, Corbin JD. Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action. Crit Rev Clin Lab Sci 1999; 36:275-328.

8. Kandel ER. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. Mol Brain 2012; 5:14. doi: 10.1186/1756-6606-5-14.

9. Lee D. Global and local missions of cAMP signaling in neural plasticity, learning, and memory. Front Pharmacol 2015; 6:161. doi: 10.3389/fphar.2015.00161.

10. Li YF, Cheng YF, Huang Y, Conti M, Wilson SP, O'Donnell JM, Zhang HT. Phosphodiesterase-4D knock-out and RNA interference-mediated knock-down enhance memory and increase hippocampal neurogenesis via increased cAMP signaling. J Neurosci. 2011; 31:172-83. doi: 10.1523/JNEUROSCI.5236-10.2011.

11. Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? Front Mol Neurosci 2010; 3:1. doi: 10.3389/neuro.02.001.2010.

12. Richter W, Menniti FS, Zhang HT, Conti M. PDE4 as a target for cognition enhancement. Expert Opin Ther Targets 2013; 17:1011-1027. doi:10.1517/14728222.2013.818656

13. Callaghan CK, O'Mara SM. Long-term cognitive dysfunction in the rat following docetaxel treatment is ameliorated by the phosphodiesterase-4 inhibitor, rolipram. Behav Brain Res 2015; 290:84-9. doi: 10.1016/j.bbr.2015.04.044.

14. Jindal A, Mahesh R, Bhatt S. Type 4 phosphodiesterase enzyme inhibitor, rolipram rescues behavioral deficits in olfactory bulbectomy models of depression:

Involvement of hypothalamic-pituitary-adrenal axis, cAMP signaling aspects and antioxidant defense system. Pharmacol Biochem Behav 2015; 132:20-32. doi: 10.1016/j.pbb.2015.02.017.

15. Wells JN, Wu YJ, Baird CE, Hardman JG. Phosphodiesterases from porcine coronary arteries: inhibition of separated forms by xanthines, papaverine, and cyclic nucleotides. Mol Pharmacol 1975; 11, 775-783.

16. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev 2006; 58:488-520.

17. Miwa S, Sugimoto N, Shirai T, Hayashi K, Nishida H, Ohnari I, Takeuchi A, Yachie A, Tsuchiya H. Caffeine activates tumor suppressor PTEN in sarcoma cells. Int J Oncol 2011; 39:465-72. doi: 10.3892/ijo.2011.1051

18. Saito T, Sugimoto N, Ohta K, Shimizu T, Ohtani K, Nakayama Y, Nakamura T, Hitomi Y, Nakamura H, Koizumi S, Yachie A. Phosphodiesterase inhibitors suppress Lactobacillus casei cell-wall-induced NF-κB and MAPK activations and cell proliferation through protein kinase A--or exchange protein activated by cAMP-dependent signal pathway. ScientificWorldJournal 2012; 2012:748572. doi: 10.1100/2012/748572

19. Sugimoto N, Miwa S, Hitomi Y, Nakamura H, Tsuchiya H, Yachie A. Theobromine,

the primary methylxanthine found in Theobroma cacao, prevents malignant glioblastoma proliferation by negatively regulating phosphodiesterase-4, extracellular signal-regulated kinase, Akt/mammalian target of rapamycin kinase, and nuclear factor-kappa B. Nutr Cancer. 2014; 66:419-23. doi: 10.1080/01635581.2013.877497.

20. Comerford KM, Lawrence DW, Synnestvendt K, Levi BP, Colgan SP. Role of vasodilator-stimulated phosphoprotein in PKA-induced changes in endothelial junctional permeability. FASB J 2002; 16: 583-585. doi: 10.1096/fj.01-0739fje

21. Loza MJ, Foster S, Peters SP, Penn RB. Beta-agonists modulate T-cell functions via direct actions on type 1 and type 2 cells. Blood 2006; 107: 2052-2060. doi:10.1182/blood-2005-08-3265

22. Sugimoto N, Miwa S, Ohno-Shosaku T, Tsuchiya H, Hitomi Y, Nakamura H, Tomita K, Yachie A, Koizumi S. Activation of tumor suppressor protein PTEN and induction of apoptosis are involved in cAMP-mediated inhibition of cell number in B92 glial cells. Neurosci Lett 2011; 497:55-9. doi: 10.1016/j.neulet.2011.04.028

23. Yoneda M, Tabata Y, Echigo R, Kikuchi Y, Ohno-Shosaku T. Availability of three-lever operant task as mouse model for studying motor sequence and skill learning. J Tsuruma Health Sci Soc Kanazawa Univ 2016; 39:113-123.

24. Unno K, Yamamoto H, Maeda K, Takabayashi F, Yoshida H, Kikunaga N, Takamori N, Asahina S, Iguchi K, Sayama K, Hoshino M. Protection of brain and pancreas from high-fat diet: effects of catechin and caffeine. Physiol Behav 2009; 96:262-9. doi: 10.1016/j.physbeh.2008.10.009.

25. Ptolemy AS, Tzioumis E, Thomke A, Rifai S, Kellogg M. Quantification of theobromine and caffeine in saliva, plasma and urine via liquid chromatography-tandem mass spectrometry: a single analytical protocol applicable to cocoa intervention studies. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 878:409-16. doi: 10.1016/j.jchromb.2009.12.019.

26. Sugimoto N, Matsuzaki K, Ishibashi H, Tanaka M, Sawaki T, Fujita Y, Kawanami T, Masaki Y, Okazaki T, Sekine J, Koizumi S, Yachie A, Umehara H, Shido O. Upregulation of aquaporin expression in the salivary glands of heat-acclimated rats. Sci Rep 2013; 3:1763. doi: 10.1038/srep01763.

27. Mitchell ES, Slettenaar M, vd Meer N, Transler C, Jans L, Quadt F, Berry M. Differential contributions of theobromine and caffeine on mood, psychomotor performance and blood pressure. Physiol Behav 2011; 104:816-22. doi: 10.1016/j.physbeh.2011.07.027.

28. Judelson DA, Preston AG, Miller DL, Muñoz CX, Kellogg MD, Lieberman HR.

518 Effects of theobromine and caffeine on mood and vigilance. J Clin
519 Psychopharmacol 2013; 33:499-506. doi: 10.1097/JCP.0b013e3182905d24.
520 29. Papadimitriou A, Silva KC, Peixoto EB, Borges CM, Lopes de Faria JM, Lopes de
521 Faria JB. Theobromine increases NAD^+/Sirt-1 activity and protects the kidney
522 under diabetic conditions. Am J Physiol Renal Physiol 2015; 308:F209-25. doi:
523 10.1152/ajprenal.00252.2014
524 30. Liu X, Smith BJ, Chen C, Callegari E, Becker SL, Chen X, Cianfrogna J, Doran AC,
525 Doran SD, Gibbs JP, Hosea N, Liu J, Nelson FR, Szewc MA, Van Deusen J. Use of
526 a physiologically based pharmacokinetic model to study the time to reach brain
527 equilibrium: an experimental analysis of the role of blood-brain barrier permeability,
528 plasma protein binding, and brain tissue binding. J Pharmacol Exp Ther 2005;
529 313:1254-62.
530 31. Yamamoto T, Yoshizawa K, Kubo S, Emoto Y, Hara K, Waters B, Umehara T,
531 Murase T, Ikematsu K. Autopsy report for a caffeine intoxication case and review of
532 the current literature. J Toxicol Pathol 2015; 28:33-6. doi: 10.1293/tox.2014-0044.
533 32. Laurent C, Eddarkaoui S, Derisbourg M, Leboucher A, Demeyer D, Carrier S,
534 Schneider M, Hamdane M, Müller CE, Buée L, Blum D. Beneficial effects of
535 caffeine in a transgenic model of Alzheimer's disease-like tau pathology. Neurobiol

536 Aging 2014; 35:2079-90. doi: 10.1016/j.neurobiolaging.2014.03.027

537 33. Almutairi MM, Gong C, Xu YG, Chang Y, Shi H. Factors controlling permeability
538 of the blood-brain barrier. Cell Mol Life Sci 2016; 73:57-77. doi:
539 10.1007/s00018-015-2050-8

540 34. Zhao J, Hao J, Fei X, Wang X, Hou Y, Deng C. Isoflurane inhibits occludin
541 expression via up-regulation of hypoxia-inducible factor 1α. Brain Res 2014;
542 1562:1-10. doi: 10.1016/j.brainres.2014.03.025

543 35. Iadecola C, Nedergaard M. Glial regulation of the cerebral microvasculature. Nat
544 Neurosc 2007; 10:1369-1376. doi:10.1038/nn2003.

545 36. Cao Y, Ni C, Li Z, Li L, Liu Y, Wang C, Zhong Y, Cui D, Guo X. Isoflurane
546 anesthesia results in reversible ultrastructure and occludin tight junction protein
547 expression changes in hippocampal blood-brain barrier in aged rats. Neurosci Lett
548 2015; 587:51-6. doi: 10.1016/j.neulet.2014.12.018

549 37. Hutchins BI. Competitive outgrowth of neural processes arising from long-distance
550 cAMP signaling. Sci Signal. 2010; 3:jc1. doi: 10.1126/scisignal.3118jc1.

551 38. Nicol X, Gaspar P. Routes to cAMP: shaping neuronal connectivity with distinct
552 adenylate cyclases. Eur J Neurosci 2014; 39:1742-51. doi: 10.1111/ejn.12543.

553 39. Ruangkittisakul A, Sharopov S, Kantor C, Kuribayashi J, Mildenberger E,

554 Luhmann HJ, Kilb W, Ballanyi K. Methylxanthine-evoked perturbation of
555 spontaneous and evoked activities in isolated newborn rat hippocampal networks.
556 Neuroscience 2015; 301:106-20. doi: 10.1016/j.neuroscience.2015.05.069.
557 40. Jabaris SS, Sumathy H, Girish R, Narayanan S, Sugumar M, Saravana Babu C,
558 Thanikachalam S, Thanikachalam M. Phosphodiesterase-4 inhibitors ameliorates
559 cognitive deficits in deoxycorticosterone acetate induced hypertensive rats via
560 cAMP/CREB signaling system. Brain Res 2015; 1622:279-91. doi:
561 10.1016/j.brainres.2015.07.003.
562 41. Zhong Y, Zhu Y, He T, Li W, Yan H, Miao Y. Rolipram-induced improvement of
563 cognitive function correlates with changes in hippocampal CREB phosphorylation,
564 BDNF and Arc protein levels. Neurosci Lett 2016; 610:171-6. doi:
565 10.1016/j.neulet.2015.09.023.
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Table 1

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

	CN (n = 10)	TB (n = 10)	*P* value
Initial (Day0)	21.1 ± 0.4	21.9 ± 0.3	*P* = 0.15
Halfway (Day30)	24.6 ± 0.4	24.3 ± 0.4	*P* = 0.59
Final (Day60)	24.9 ± 0.5	24.5 ± 0.3	*P* = 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)	*P* value
Glucose (Day30)			
(mg/dL plasma)	254.6 ± 13.85	241.35 ± 12.91	*P* = 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	

(μg/mL cerebral cortex)	Not detected	Not detected	
BDNF (Day30)			
(pg/g hippocampus)	25.81 ± 0.85	29.60 ± 0.99	$P = 0.0086$
(pg/g cerebral cortex)	20.28 ± 0.61	22.67 ± 0.82	$P = 0.029$

Each value represents the mean ± SEM

612 **Figure legends**

613 **Figure 1**

614 Feeding and experiment schedules. The mice were divided into two groups. The mice in
615 the first group (control; CN) were fed the standard chow (CRF-1) for 30 days and were
616 then switched to CE-2 for 30 days. The second group (theobromine; TB) was fed chow
617 supplemented with 0.05% (W/W) theobromine (CRF-1 with 0.05% of theobromine) for
618 30 days and were then switched to CE-2 for 30 days. Body weight was measured on
619 Day0, Day30, and Day60. The theobromine concentrations in plasma and the cerebral
620 cortex were measured on Day30 and Day60. The plasma glucose, brain-derived
621 neurotrophic factor (BDNF), phosphorylated vasodilator-stimulated phosphoprotein
622 (VASP), and cAMP-response element-binding protein (CREB) levels were measured on
623 Day30. The Lever operant tasks were performed between Day30 and Day60.

624

625 **Figure 2**

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

629

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 ± 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 ± 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 ± 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.

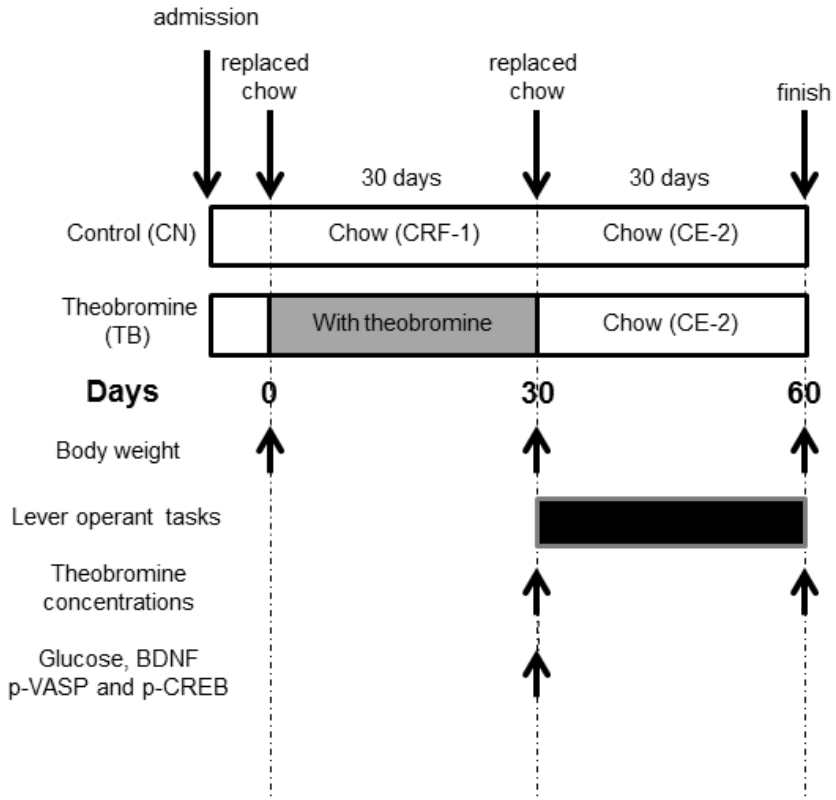

Figure 1

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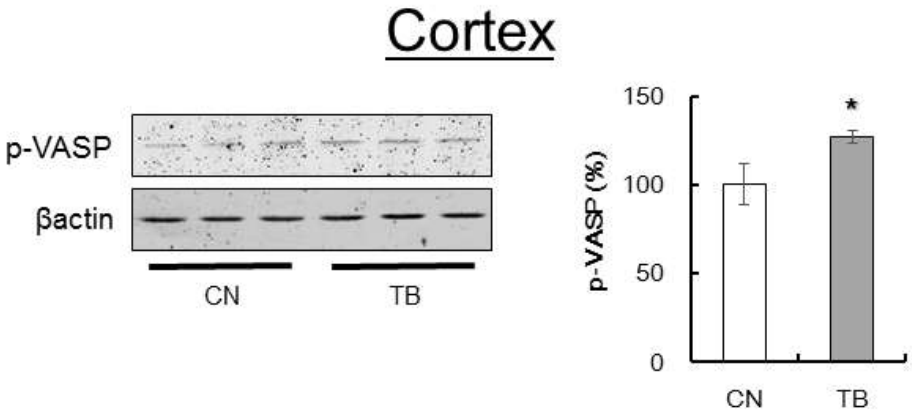

Figure 2

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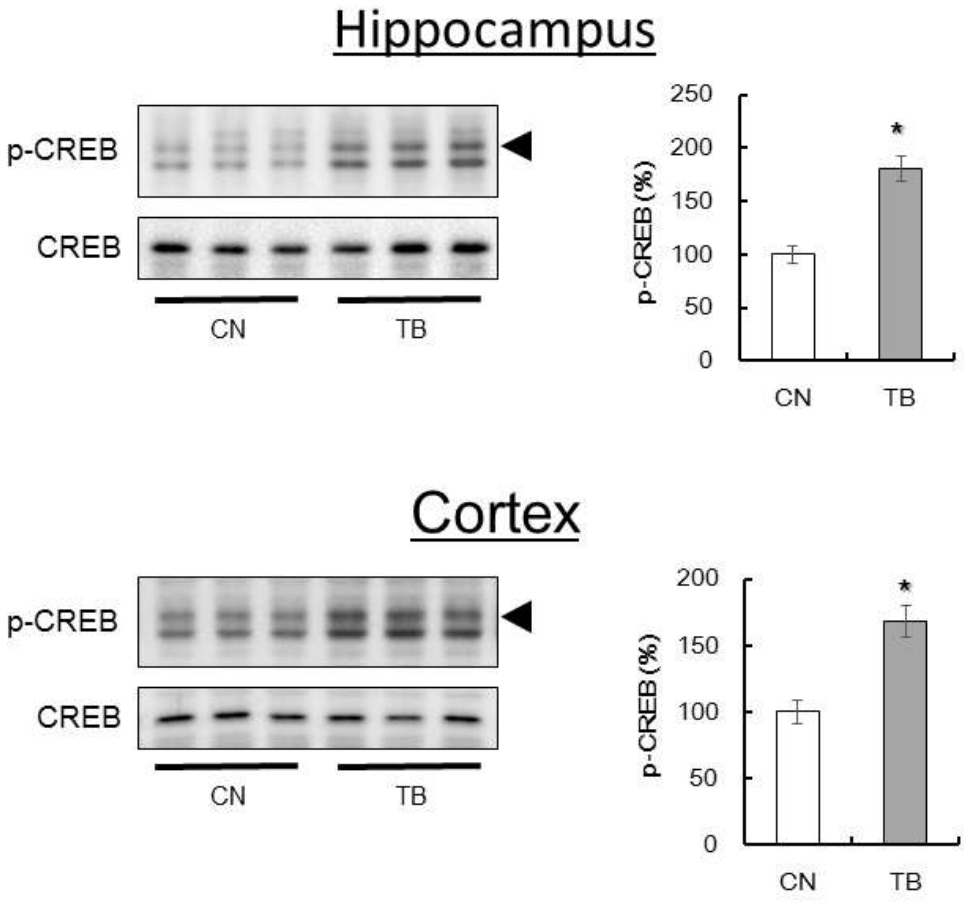

Figure 3

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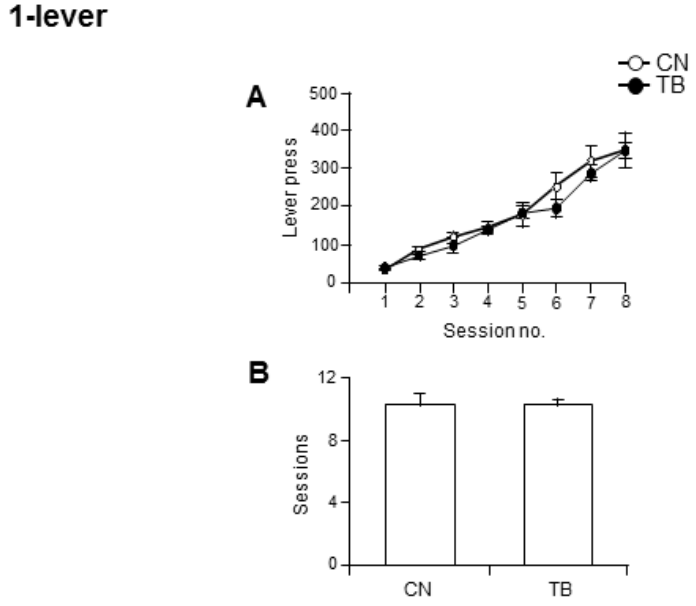

Figure 4

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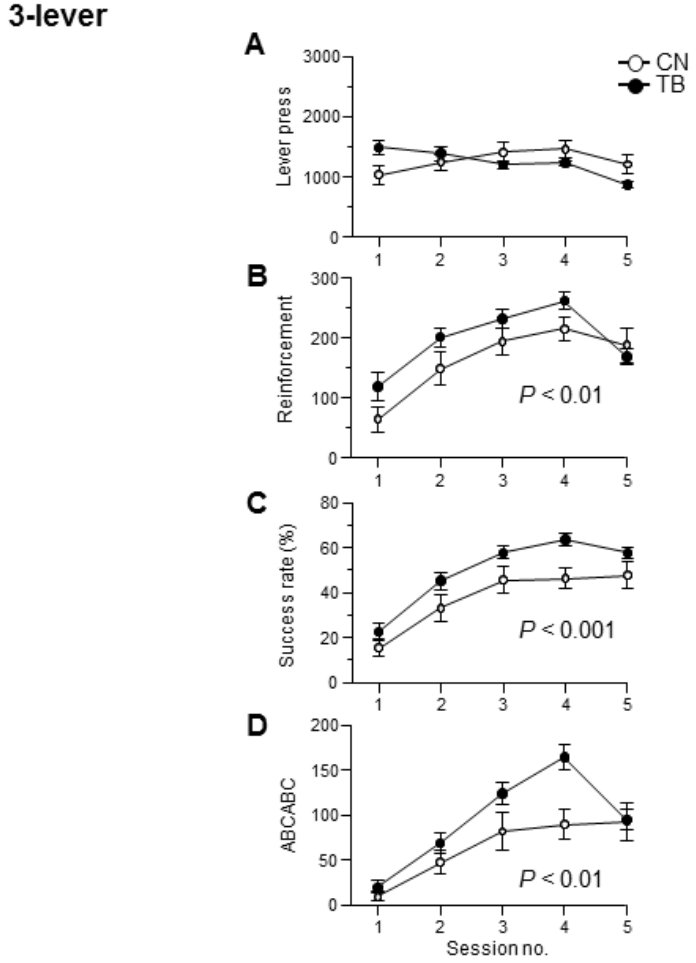

Figure 5

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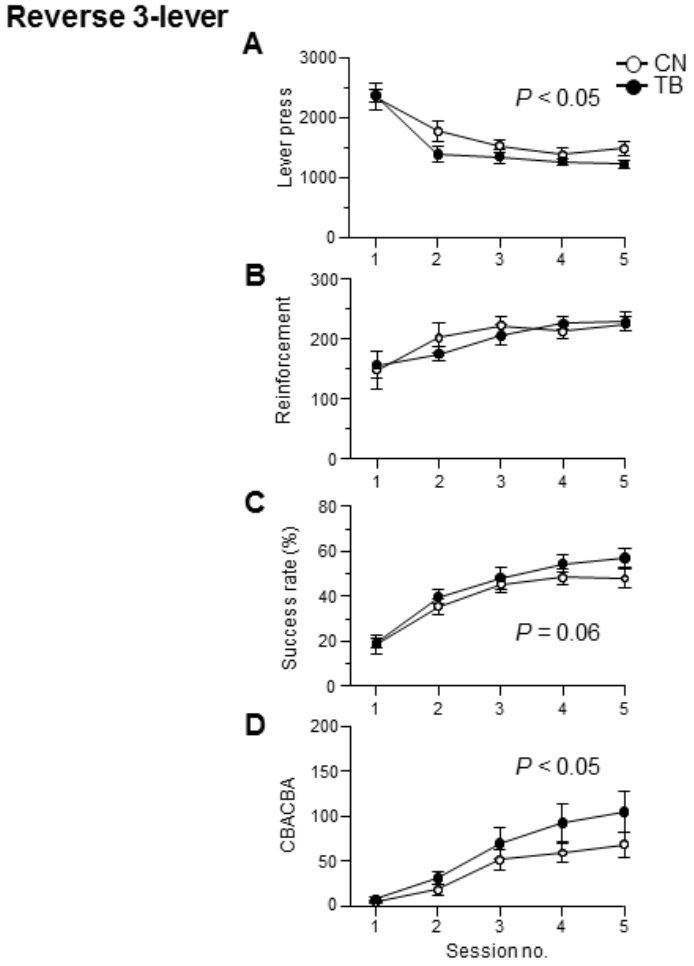
Reverse 3-lever
A
B
C
D
CN
TB
P < 0.05
P = 0.06
P < 0.05
Lever press
Reinforcement
Success rate (%)
CBACBA
Session no.

Figure 6

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