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Theobromine improves hyperactivity, inattention, and working memory via modulation of dopaminergic neural function in the frontal cortex of spontaneously hypertensive rats

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Abbreviations

AAR	Alternate arm return
AR	Adenosine receptor
ADHD	Attention-deficit/hyperactivity disorder
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BUN	Blood urea nitrogen
CN	Control
CREB	cAMP response element binding
DAT	Dopamine transporter
DAPI	4′,6-diamidino-2-phenylindole
DRDs	Dopamine receptors
ELISA	Enzyme-linked immunosorbent assay
GOT	Glutamic oxaloacetic transaminase/aspartate transaminase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
NeuN	Neuronal nuclei
OFT	Open-field test
PDB	Protein Data Bank archive
PFC	Prefrontal cortex
RBC	Red blood cell
SAR	Same arm return
SE	Standard error
SHR	Spontaneously hypertensive rat
SNAP-25	Synaptosome-associated protein-25
TB	Theobromine
TH	Tyrosine hydroxylase
TP	Total protein
UA	Urea acid
VMAT-2	Vesicular monoamine transporter-2
WBC	White blood cells
WKY	Wistar–Kyoto rats

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a developmental disorder and dopaminergic dysfunction in the prefrontal cortex (PFC) may play a role. Our previous research indicated that theobromine (TB), a methylxanthine, enhances cognitive function in rodents via the PFC. This study investigates TB's effects on hyperactivity and cognitive function in stroke-prone spontaneously hypertensive rats (SHR), an ADHD animal model. Male SHRs (6-week old) received a diet containing 0.05% TB for 40 days, while control rats received normal diets. Age-matched male Wistar–Kyoto rats (WKY) served as genetic controls. During the TB administration period, we conducted open-field tests and Y-maze tasks to evaluate hyperactivity and cognitive function, then assessed dopamine concentrations and tyrosine hydroxylase (TH), dopamine receptor D1−5 (DRD1−5), dopamine transporter (DAT), vesicular monoamine transporter-2 (VMAT-2), synaptosome-associated protein-25 (SNAP-25), and brain-derived neurotrophic factor (BDNF) expressions in the PFC. Additionally, the binding affinity of TB for the adenosine receptors (ARs) was evaluated. Compared to WKY, SHR exhibited hyperactivity, inattention and working memory deficits. However, chronic TB administration significantly improved these ADHD-like behaviors in SHR. TB administration also normalized dopamine concentrations and expression levels of TH, DRD2, DRD4, SNAP-25, and BDNF in the PFC of SHR. No changes were observed in DRD1, DRD3, DRD5, DAT, and VMAT-2 expression between SHR and WKY rats, and TB intake had minimal effect. TB was found to have affinity binding to ARs. These results indicate that long-term TB supplementation mitigates hyperactivity, inattention and cognitive deficits in SHR by modulating dopaminergic nervous function and BDNF levels in the PFC, presenting a potential adjunctive treatment for ADHD.

Keywords: Theobromine, attention-deficit/hyperactivity disorder, prefrontal cortex, spontaneously hypertensive rats

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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental disorder, diagnosed in approximately 5% of children and adolescents and 2.5% of adults.[1,2] Beyond the common symptoms of inattention, hyperactivity, and impulsivity, individuals with ADHD frequently exhibit working memory impairments.[2,3] While the etiology of ADHD remains unclear, deficits in the dopaminergic neural system within the prefrontal cortex (PFC) may contribute to the disorder.[3–6] First-line treatments for ADHD patients typically involve psychostimulants such as methylphenidate and amphetamine.[2] These medications enhance attention and executive functions by modulating dopaminergic neural transmission in the PFC. However, some patients may experience adverse effects, including anorexia, weight loss, and headache, leading to discontinuation of treatment.[7,8] Some patients cannot tolerate stimulants because of severe side effects. Overmedication and short- and long-term side effects, of stimulants are other challenges to consider.[9] Non-stimulants, such as selective noradrenaline reuptake inhibitors, have shown some clinical efficacy against ADHD symptoms; however, their side effects such as increased blood pressure, tachycardia, and liver dysfunction, raise concerns regarding their use.[10] Therefore, the development of ADHD treatments without adverse effects is long awaited.

Several factors related to changes in the dopaminergic system are of interest as potential therapeutic targets for ADHD. Neural development and plasticity are considered significant targets in ADHD's pathogenesis.[11] Neurotrophic factors, crucial for neurodevelopment and synaptic plasticity in the brain, have been linked to ADHD development due to their abnormalities.[12] Specifically, the brain-derived neurotrophic factor (BDNF), involved in the nervous system's synaptic plasticity, is a potential biological target for ADHD treatment.[13] Genetic association research suggests that disruption in neuroplasticity mechanisms contributes to ADHD's pathophysiology.[12] For instance, gene polymorphisms and functional abnormalities of dopamine receptors (DRDs), dopamine transporter (DAT), and vesicular monoamine transporter 2 (VMAT-2) are associated with attention function and impulse control in ADHD.[14,15] Furthermore, DNA mutations in synaptosomal-associated protein 25 (SNAP-25), a presynaptic plasma membrane protein integral to synaptic transmission with syntaxin 1A, may lead to reduced transcript expression and increased ADHD risk.[16-19] Adenosine receptors (ARs) are promising therapeutic targets for ADHD because they mediate central dopaminergic function.[20] There are different types of ARs including A1R, A2AR, A2BR and A3AR, characterized by seven transmembrane domains. AR antagonists can reduce ADHD symptoms in humans and animal models.[20]

The majority of research on potential treatments to mitigate ADHD symptoms relies on animal models. While no animal model can perfectly reproduce all the pathological symptoms of ADHD, stroke-prone spontaneously hypertensive rats (SHRs) are among the most frequently used animal models for ADHD.[21,22] SHR exhibit pathological features similar to those observed in patients with

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ADHD, including hyperactivity, inattention, and deficits in working memory.[21-25] In addition, SHRs have neuropathological changes similar to those observed in patients with ADHD. For example, SHRs showed altered dopaminergic function and function of proteins, such as DAT, DRD, and SNAP-25, compared with the genetic control rats.[17,21-25]

Compounds derived from natural sources have shown potential in ameliorating central nervous system disorders, including ADHD.[26,27] Theobromine (TB), a primary methylxanthine found in cacao beans and prevalent in cacao products like cocoa and dark chocolate, is one such compound.[28] Our previous research indicated that long-term TB supplementation enhances motor learning and working memory by increasing BDNF levels in the PFC of rodents[29,30] Additionally, chronic consumption of dark chocolate, rich in TB, has been reported to improve frontal lobe function in healthy adolescents.[31] However, the effects of TB administration on ADHD traits remain unexplored to the best of our knowledge. This study, therefore, investigates the impact of TB on hyperactivity, inattention, cognitive performance, and other pathological features in SHR, a commonly used animal model of ADHD. Additionally, bioinformatics studies were also conducted to determine whether TB binds to ARs.

Materials and methods

Animals

Male SHRSP/Ezo rats (SHR, n = 20) aged 6 weeks (body weight 130–135 g) were procured from Japan SLC Inc. (Hamamatsu, Japan). They were kept in an environment with a temperature of 25°C ± 0.8°C and a 12:12-h light-dark cycle (lights turned on at 7:00 h), with unrestricted access to food and water. Age-matched male Wistar–Kyoto rats (WKY, n = 16) served as genetic controls. Rats were kept in plastic cages lined with wood chips, 3–4 rats each, and the cages were changed once a week. All animal experiments adhered to the Guidelines for Animal Experimentation of the Shimane University Faculty of Medicine, which were derived from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. The study protocol was approved by the Committee on the Ethics of Animal Experiments of Shimane University (Approval No. IZ3-47).

Experimental schedule

SHR and WKY were given unrestricted access to standard chow (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water for a week. Following this, both SHR and WKY rats were randomly divided into two groups: control rats (CN) continued on the CRF-1, and TB-administered rats were fed a standard CRF-1 diet supplemented with 0.05% (W/W) TB (Oriental Yeast Co., LTD.) for a period of 40 days. This resulted in the formation of four experimental groups: WKY-CN (n = 8), WKY-TB (n = 8), SHR-CN (n = 10), and SHR-TB (n = 10). The dosage and duration of TB

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administration were determined based on prior studies.[29,30,32] Our previous studies have shown that long-term administration of 0.05% TB enhances motor learning through the activation of the cAMP response element binding protein (CREB)/BDNF signaling cascade in mouse PFC.[29] Upon long-term administration in rats, TB crossed the blood–brain barrier and acted on the PFC to increase spatial cognitive function without adverse effects.[30,32] Body weight was measured three times, and food intake volume was measured and averaged every 10 days throughout the experimental period.

Open-field test (OFT)

OFT is one of the most common assessments to estimate locomotor activity in rodents and is widely used to assess hyperactivity in animal models of ADHD.[17,24] To assess hyperactivity, an OFT was conducted 36−37 days post-initiation of TB administration. This test utilized a two-level infrared beam apparatus (Scanet MV-40; Melquest, Toyama, Japan), an automated system designed to measure locomotor activity. A day prior to the test, the rats were acclimatized to the apparatus. The OFT was carried out from 9:00 to 14:00 under low-light conditions (<75 lux) in a quiet room. Rats were placed at the center of the open-field (44 × 44 × 30 cm) and allowed to explore for a duration of 6 min. The quantity of horizontal movement (locomotion) and vertical activity (i.e., rearing) was automatically recorded as the number of times the beam was interrupted. Between trials, any odors and animal waste were eliminated by disinfecting the area with 70% ethanol.

Y-maze test

The Y-maze test is frequently used to assess ADHD characteristics because this task can assess locomotor activity, attentional function and working memory in rodents.[33,34] The test was conducted 38−39 days post-initiation of TB administration. A day prior to the test, rats were acclimatized to the apparatus. The test was carried out between 9:00 and 14:00. The apparatus comprises three identical arms (45 × 12 × 35 cm) diverging at a 120° angle, forming an equilateral triangular central area. Each rat was placed at the center of the maze and allowed to explore for 8 min. Rats typically alternate visits between the three arms, tending to explore the least recently visited arm.[33,34] Efficient alternation requires rats to utilize their working memory by maintaining a record of the most recently visited arms and continuously updating this record.[35] Behavior was video-recorded for subsequent analysis. An arm entry was recorded when the rat placed all four paws within an arm. The percentage of spontaneous alternations was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus one), multiplied by 100. The percentages of alternate arm return (AAR) and same arm return (SAR) were also scored to assess aspects of attention within spontaneous exploratory behavior.[36]

Blood and PFC collection

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Forty days post-initiation of TB administration, the rats were deeply anesthetized via isoflurane inhalation (Pfizer, NY, USA). Blood was then drawn from the rats' right ventricle and collected in sterile tubes containing sodium heparin (Mochida, Tokyo, Japan). Following this, saline was perfused transcardially, and the brains were swiftly removed from the skull. For Western blotting and enzyme-linked immunosorbent assay (ELISA), the PFC (n = 5 or 6), encompassing the prelimbic, infralimbic, and anterior cingulate cortices, was promptly dissected from a coronal section as per the brain atlas.[37] The PFC samples were instantly frozen on liquid nitrogen and stored at −80°C until further use. For immunohistochemical analysis, the brains from each group (n = 3 or 4) were immersed in 10 N Mildform (Fujifilm Wako Pure Chemical) and stored at 4°C for 24 h.

Western blot analysis

The PFCs were homogenized using a glass homogenizer in lysis buffer. This buffer contained 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 10 mM Tris-HCl (pH 7.6), and 1× protease inhibitor cocktail (Fujifilm Wako Pure Chemical). The homogenized samples underwent sonication and were then centrifuged at 12,000 × g for 15 min at 4°C. The supernatants were collected and analyzed via Western blotting, following the method described previously.[38,39] The protein concentrations in the extracts were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of these protein extracts were boiled in a 6 × SDS sample buffer (Nacalai, Kyoto, Japan) and then separated by 7.5-12.5% SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). These membranes were blocked with 5% skim milk and then incubated overnight at 4°C with primary antibodies. These antibodies included polyclonal rabbit antityrosine hydroxylase (TH, 1:1000; CellSignaling, Danvers, MA, USA), polyclonal rabbit anti-DAT, anti-VMAT-2, polyclonal rabbit antidopamine receptor (DRD) 1−5 (1:1000; Proteintech, USA), polyclonal rabbit anti-SNAP-25, polyclonal rabbit antisyntaxin, and polyclonal rabbit anti-synapsin-1 (1:1000; CellSignaling). To ensure that equal amounts of protein were loaded, a monoclonal mouse anti-GAPDH antibody (1:5000; Cell Signaling) was used. After washing, the membranes were incubated with horseradish peroxidase-linked antirabbit or antimouse secondary antibody (1:2000; CellSignaling, Danvers, MA, USA) for 2 h. The membranes were then developed using chemiluminescent substrate (SuperSignal West Pico PLUS, Thermo Fisher Scientific) and visualized using an Amersham ImageQuant800 (Cytiva, MA, USA).

ELISA

The concentrations of dopamine, TH, and BDNF in the PFC were determined using a Dopamine ELISA Kit (Enzo Life Sciences, NY, USA), the Rat Tyrosine Hydroxylase ELISA Kit (MyBioSource, San Diego CA, USA), and the BDNF ELISA Kit (MyBioSource). These

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measurements were performed according to the protocols provided by the manufacturers. The absorbance of the samples was measured using a DTX880 multimode microplate reader (Beckman Coulter, Pasadena, CA, USA). The protein concentrations in the samples were then calculated using the SoftMax Pro software (Molecular Devices, LLC, San Jose, CA, USA). This method of calculation is consistent with the one described previously.[38]

Immunohistochemistry

Whole brains (n = 3−4) were fixed and then immersed in a 20% (w/v) sucrose solution. Brain sections, 20-μm-thick, were prepared using a cryostat CM1520 (Leica, Wetzlar, Germany). These sections were incubated in a 10 mM sodium citrate buffer (pH 6.0) and blocked with 3% normal goat serum (Agilent, Santa Clara, CA, USA). The brain sections were then incubated overnight at 4°C with primary antibodies. The primary antibodies used in this study were monoclonal rabbit anti-TH (1:500) and mouse anti-neuronal nuclei (NeuN, 1:500, CellSignaling). Alexa Fluor 488-conjugated antirabbit IgG and Alexa Fluor 594-conjugated antimouse IgG (1:500; Molecular Probes, OR, USA) were used as the secondary antibodies. To detect cell nuclei, the sections were counterstained with a 4′,6-diamidino-2-phenylindole (DAPI) solution (1:2000, Dojindo, Kumamoto, Japan). After staining, the sections were washed and covered with 80% glycerol. All sections were visualized under ×20 magnification using an FV-1000D confocal microscope (Olympus, Tokyo, Japan) and Fluoview imaging software (Olympus), as described previously[40,41] The average intensity for TH-expressing axons was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Blood cell count and biochemistry

To assess the potential adverse effects of long-term TB administration, blood cell counts and biochemical tests were conducted. The counts for white blood cells (WBC), red blood cell (RBC), and platelets (PLT), as well as the levels of hemoglobin (HGB), hematocrit (HTC), and mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC) were measured using a KX-21NV device (Sysmex, Hyogo, Japan), following the method described previously.[42] Plasma was isolated and tested to quantitatively determine ten parameters: glutamate–pyruvate transaminase/alanine transaminase (GPT), γ-glutamyl transpeptidase (γGT), glutamic oxaloacetic transaminase/aspartate transaminase (GOT), total cholesterol (T-Cho), triglyceride (TG), albumin (Alb), total protein (TP), urea acid (UA), creatinine (Cre), and blood urea nitrogen (BUN). These tests, which assess liver and kidney function, were performed using the automated biochemical analyzer Spotchem EZ SP-4430 (Arkray, Kyoto, Japan). The Spotchem EZ Reagent Strip KENSHIN-2 and Spotchem EZ Reagent Strip Kidney-3 (both from Arkray) were used for these tests, as described previously.[42]

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In-silico molecular docking simulation study

Information on the protein structure of ARs was obtained from the Protein Data Bank (PDB) archive. The most similar co-crystallized PDB structures of ARs, namely 6D9H,[43] 2YDO,[44] and 3EML,[45] were used to perform docking simulation studies for A1AR, A2AR, and A3AR, respectively. The caffeine co-crystallized structure of A2AR (PDB ID: 3RFM)[46] was obtained from the RCSB PDB data repository. Subsequently, the ligands of these receptors were separated from the binding pockets, and the pockets were docked with TB (ligand) and other drugs (with alleged roles as agonists and antagonists). This *in-silico* study used the binding of adenosine (natural ligand of ARs) and established agonists and antagonists (modulators) of A1AR, A2AR, and A3AR. We selected *N6*-cyclyhexyladenosine (A1AR agonist),[47] rolofylline (A1AR antagonist),[48] regadenoson (A2AR agonist),[49] istradefylline (A2AR antagonist),[50] IB-MECA (A3AR agonist),[51] and MRS-1334 (A3AR antagonist).[52,53] The binding of TB was then compared with that of the agonists and antagonists, interims of binding energy, H-bond formation, and steric interactions with the surrounding amino acid residues. Typically, for binding energy or binding affinity, the more negative the energy, the better the ligand. The canonical SMILES of these compounds were retrieved from the NCBI PubChem database and fed into Marvin Sketch 5.10v in Chemaxon to convert them to their 3D conformers. Using the MMFF94 force field method, the lowest energy conformers of the ligands were selected for docking studies. Molegro Virtual docker (MVD, free license version) was used for docking studies. The 3D structures of docked ligands and their respective ARs were visualized using Chimera (v 17.1).

Calculation of sample size power

Sample size was calculated using PS: Power & Sample Size Calculation Software Version 3.1.2 (Vanderbilt University, Nashville, TN, USA). The primary outcomes of this study were the effects of TB intake on the change in working memory in SHR. Assuming that the standard deviation was not greater than twice the effect of TB supplementation,[30] a bilateral alpha of 95%, and power 90%, the number of rats was 10 per group.

Statistical analysis

The data are presented as the mean ± standard error (SE). The statistical analyses were conducted using the SPSS software, version 24.0 (IBM Corp., Armonk, NY, USA). The normality of the data was assessed using the Shapiro–Wilk normality test. The data were considered to follow a normal distribution when $\alpha = 0.05$ and $p > 0.05$. Behavioral, biochemical, and histological data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests. Body weight and food intake volume were analyzed by two-way (time × group) ANOVA, followed by Tukey's post-hoc tests. Correlations between behavioral and biochemical data were evaluated using

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the Pearson's correlation coefficient. All statistical tests were two-tailed, and a p-value of less than 0.05 was considered to indicate statistical significance.

Results

OFT

A one-way ANOVA revealed significant differences in locomotion counts among the groups (Fig. 1A, $F_{(3,32)} = 41.403$, $p < 0.001$). The locomotion counts in the SHR-CN group were significantly higher than those in the WKY-CN group ($p < 0.001$), indicating that the SHR group was more hyperactive than the WKY group. However, the locomotion counts in the SHR group were significantly reduced by TB supplementation (Fig. 1A, $p < 0.001$). There was no significant difference in the locomotion counts between the WKY-CN and WKY-TB groups (Fig. 1A, $p = 0.962$). These results indicate that TB supplementation can alleviate hyperactivity in SHR. The number of rearings was also counted, and significant group differences were observed (Supplemental Fig. 1, $F_{(3,32)} = 11.465$, $p < 0.01$). However, the administration of TB did not significantly alter the number of rearings in either the WKY ($p > 0.05$) or SHR ($p > 0.05$) groups.

Y-maze test

In the Y-maze test, significant differences were observed in the number of arm entries among the groups (Fig. 2A, $F_{(3,32)} = 3.850$, $p = 0.005$). The SHR-CN group had a significantly higher number of arm entries compared to the WKY-CN group (Fig. 2A, $p = 0.004$). However, TB administration significantly reduced the number of arm entries in the SHR group (Fig. 2A, $p = 0.012$), indicating that TB improved the hyperactivity of SHR. The percentages of spontaneous alternation, which is a measure of working memory and attentional function, also showed significant differences among the groups (Fig. 2B, $F_{(3,32)} = 8.498$, $p = 0.003$). Chronic TB intake improved these percentages in the SHR group (Fig. 2B, $p = 0.030$). While TB intake improved the AAR, it did not reach statistical significance (Fig. 2C, $F_{(3,32)} = 1.782$, $p > 0.05$). The SAR differed significantly among the groups (Fig. 2D, $F_{(3,32)} = 5.282$, $p = 0.026$). The SAR was significantly increased in the SHR-CN group compared to the WKY-CN group (Fig. 2D, $p < 0.001$). However, TB ingestion significantly reduced the SAR in both the WKY ($p < 0.05$) and SHR ($p = 0.016$) groups. These results indicate that TB supplementation may improve attention and working memory in rats.

Dopamine concentration and protein expression of dopaminergic neurotransmission factors

In line with the dopamine deficit theory, we assessed the concentration of dopamine and factors related to dopaminergic neurotransmission in the PFC. We observed significant group differences in dopamine concentrations in the PFC (Fig. 3A, $F_{(3,18)} = 4.565$, $p = 0.017$). TB administration notably increased dopamine concentrations in the PFC of the SHR group ($p = 0.020$) but not in the WKY

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group ($p > 0.05$). Using ELISA, we found that the concentration of TH, a rate-limiting enzyme for dopamine synthesis, in the PFC was significantly different between groups (Fig. 3B, $F_{(3,18)} = 20.09$, $p < 0.001$). The TH concentrations in the SHR-CN group were drastically lower than those in the WKY-CN group ($p < 0.001$). However, TB intake normalized these concentrations ($p < 0.001$). Western blot analysis revealed that the expression of TH in the PFC was markedly decreased in the SHR-CN group compared with the WKY-CN group (Fig. 3C, $F_{(3,18)} = 53.628$, $p < 0.001$). However, this expression significantly improved with TB intake ($p < 0.001$). For dopamine receptors, the expression of DRD2 and DRD4 in the PFC was significantly different between the groups (Fig. 3C, DRD2: $F_{(3,18)} = 8.032$, $p < 0.001$, DRD4: $F_{(3,18)} = 12.426$, $p < 0.05$). Both DRD2 and DRD4 expression levels were significantly lower in the SHR-CN group than in the WKY-CN group (DRD2, $p < 0.01$, DRD4, $p < 0.05$). However, TB intake improved their expression levels in the PFC ($p < 0.01$). A similar result was observed in DRD1, but it did not reach statistical significance (Fig. 3C, $F_{(3,18)} = 0.428$, $p > 0.05$). The expression levels of DRD3 ($F_{(3,18)} = 0.254$, $p > 0.05$), DRD5 ($F_{(3,18)} = 0.361$, $p > 0.05$), DAT ($F_{(3,18)} = 0.262$, $p > 0.05$), and VMAT-2 ($F_{(3,18)} = 0.244$, $p > 0.05$) did not change among the four groups (Fig. 3C and Supplemental Fig. 2).

TH immunostaining

To validate the results obtained from Western blotting and ELISA, changes in TH expression in the PFC were investigated through immunostaining. The findings revealed a reduction in TH-positive fibers in the SHR-CN group compared to the WKY-CN group. This indicates that the dopaminergic projection from the midbrain and/or dopamine synthesis in the PFC may be impaired in the SHR group compared to the WKY group. However, a higher number of TH-positive signals were observed in the SHR-TB group than in the SHR-CN group. A quantitative analysis of the mean integral optical density showed group differences in TH immunoreactivities (Fig. 4, $F_{(3,10)} = 31.020$, $p < 0.001$), with significant differences observed between the SHR-CN and SHR-TB groups ($p < 0.001$). The number of NeuN-immunopositive cells did not vary between groups. As an additional analysis, TH expression levels in the striatum were also examined. The expression levels of TH were significantly lower in the striatum of the SHR group than in the WKY group (Supplementary Fig. 3). Interestingly, long-term TB administration to the SHR group increased TH expression levels in the striatum (Supplementary Fig. 3). This indicates that TB may play a role in activating the dopaminergic nervous system, including the mesolimbic and mesocortical pathways.

Protein expression of synapses-related factors

To assess presynaptic protein expression levels in the PFC of SHR, SNAP-25, syntaxin-1, synapsin-1, and synaptophysin proteins were measured by Western blotting, as shown in Figure 5. Significant group differences were observed in the levels of SNAP-25 in the PFC (Fig. 5, $F_{(3,18)} = 4.245$, $p <$

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0.001). The SHR group exhibited lower levels of SNAP-25 in the PFC compared to the WKY group ($p < 0.01$), but these levels were increased by TB administration ($p < 0.01$). Similar results were obtained for the expression of syntaxin 1A in the PFC (Fig. 5, $F_{(3,18)} = 4.323$, $p = 0.018$). The expression of synapsin-1 in the PFC also showed significant group differences (Fig. 5, $F_{(3,18)} = 2.704$, $p = 0.042$). The expression levels of synapsin-1 in the SHR-TB group were significantly higher than those in the SHR-CN group ($p < 0.05$). In the PFC, synaptophysin expression was slightly lower in SHR than WKY, and a trend was observed toward a slight increase with TB administration to SHR, which was not statistically significant (Fig. 5, $F_{(3,18)} = 0.846$, p = 0.162).

BDNF levels in the PFC

To verify the impact of TB on dopamine neurodevelopment and neuroplasticity, we analyzed the expression levels of the neurotrophic factor BDNF using an ELISA. A one-way ANOVA revealed significant group differences in BDNF concentration in the PFC (Fig. 1A, $F_{(3,18)} = 4.302$, $p < 0.01$). Administration of TB increased BDNF expression in the PFC in both the SHR ($p < 0.005$) and WKY ($p < 0.05$) groups.

Correlation analysis

Locomotor activity of SHR showed a significant negative correlation with the expression of TH (r = −0.681, $p < 0.001$), DRD2 (r = −0.684, $p < 0.001$), DRD4 (r = −0.504, $p < 0.017$), and SNAP-25 (r = −0.659, $p < 0.001$) in the PFC, but not significantly with BDNF (r = −0.121, $p = 0.220$) (Supplemental Table S1). The spontaneous alternation (%) in SHR showed a significant positive correlation with the expression of TH (r = 0.599, $p = 0.003$), DRD2 (r = 0.643, $p = 0.004$), DRD4 (r = 0.393, $p = 0.020$), SNAP-25 (r = 0.649, $p < 0.001$), and BDNF (r = 0.315, $p = 0.045$) in the PFC (Supplemental Table 1).

Body weight, food intake, blood counts, and blood biochemical findings

Body weight at 0, 20, and 40 days during the experimental period is detailed in Table 1. Rats in both groups exhibited no significant differences in body weight on day 0 ($p = 0.789$), day 20 ($p = 0.898$), or day 40 ($p = 0.632$). Food intake volumes at 10, 20, 30, and 40 days of the administration period are shown in Table 2. While there were no significant differences in dietary intake at days 10 ($p = 0.732$), 20 ($p = 0.812$), 30 ($p = 0.683$), and 40 ($p = 0.147$), there was a slight decrease in the SHR group compared to the WKY group. Blood cell counts and blood biochemistry data are summarized in Table 3 and Table 4, respectively. No significant differences were observed in blood counts (WBC, RBC, PLT, HGB, HTC, MCH, and MCHC, Table 3) or biochemical findings (GOT, GPT, gGT, TG, T-Cho, Cre, Alb, TP, UA, and BUN, Table 4). These results indicate that prolonged TB ingestion does not lead to serious adverse effects on hepatic and renal function, feeding, body

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weight, or blood cell findings in both SHR and WKY.

In-silico binding analysis of TB with ARs

Ligand poses with corresponding binding energy and/or binding affinity, namely the MolDock score, are summarized in Table 5. This assay revealed that TB has affinity for A1AR, A2AR, and A3AR. For A1AR, adenosine formed hydrogen bonds with Tyr12, Glu172, and Asn254 in the binding pocket. Tyr12, Ala66, Asn70, Glu170, Glu172, and Asn254 sterically interacted at the binding site surrounding adenosine. TB interacted slightly deeper at the binding site, was more likely to form H bonds with Tyr12, Glu170, and Phe271 (Table 5), and sterically interacted with Ile69, Phe171, Tyr12, Glu170, and Tyr271. Finally, docking of TB into the binding pocket of A1AR resulted in a binding energy for TB of −69.9 kcal/mol, compared to that of adenosine, which was −98.6 kcal/mol. Expectedly, the synthetic agonist *N6*-cyclyhexyladenosine and the antagonist rolofylline had better affinities for A1AR (−118.9 and −117.3 kcal/mol, respectively). For A2AR (2YDO), TB formed H bonds with Phe168 and Glu169, and sterically interacted with Phe168, Glu169, Ile66, Ser67, and Ile274. However, while docking with A2AR, TB showed a binding energy of −64.7 kcal/mol and adenosine showed a binding energy of −110 kcal/mol (Table 5). Similarly, TB had a binding energy of −71.3 kcal/mol for A3AR, which was higher than that of adenosine (−104.3 kcal/mol) (Table 5). Interestingly, TB (−65.7 kcal/mol) had better affinity for A2AR (PDB ID: 3RFM) than caffeine (−77.1 kcal/mol) in a docking assay for the binding site of A2AR co-crystallized with caffeine (Table 5). This is because the more negative the value, the greater the affinity and vice versa. A 3D structural diagram showing the affinities of adenosine, caffeine, and TB for A2AR (PDB ID: 3RFM) is shown in Figure 7.

Discussion

This study explored the impact of TB administration on ADHD traits in SHR. As far as we are aware, this is the first investigation into the effects of TB on ADHD pathogenesis. Our findings indicate that TB administration ameliorated hyperactivity, inattention, and working memory deficits in SHR. Furthermore, TB supplementation modulated the concentration of dopamine and normalized the expression levels of dopaminergic neurotransmission factors such as TH, DRD2, DRD4, and SNAP-25 in the PFC. TB supplementation also increased the concentration of BDNF in the PFC of SHR. Collectively, these results indicate that long-term TB administration could potentially improve hyperactivity, inattention, and working memory impairment by modulating dopaminergic neurotransmission and synaptic plasticity in the PFC. Thus, TB merits further exploration as a potential treatment for ADHD.

While various mechanisms have been proposed for the development of ADHD, dysfunction in the dopamine system within the PFC stands out as one of the most supported hypotheses.[1-3,5] In this

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study, TB demonstrated an increase in dopaminergic neural transmission factors, including dopamine, TH, DRD2, and DRD4, within the PFC. These changes are speculated to have contributed to an improvement in ADHD-like behavior in SHR. The molecular mechanisms underlying the enhancement of dopaminergic neural transmission factors in SHR due to TB remain poorly understood at present. However, the compound's various pharmacological properties, notably its role as an analog of caffeine and a nonselective antagonist for ARs, may be implicated.[54] ARs are functionally associated with specific postsynaptic dopamine receptors, such as DRD2, influencing dopamine binding and exerting a stimulatory effect.[55] Specifically, the central adenosine receptor A2AR is interconnected with the dopamine neurotransmitter system and is implicated in regulating attention, executive function, and working memory.[20] This indicates a potential link with ADHD traits.[20,56] The hypothesis that AR antagonists could serve as a novel therapeutic strategy for ADHD gains strength, especially considering the use of caffeine in treating this disorder. Indeed, caffeine has demonstrated the ability to enhance attention and short-term memory in an animal model of ADHD by modulating dopaminergic neurotransmission[57-59] and has shown clinical benefits in patients with ADHD.[60,61] However, the effectiveness of caffeine in ameliorating ADHD symptoms and its potential superiority over existing psychostimulants remain subjects of controversy.[62] In an *in-silico* study, TB docked far downstream of the endogenous adenosine ligand. Compared to adenosine, TB hydrogen bonded with different amino acids in A1AR, A2AR, and A3AR. In addition, amino acids involved in steric interactions differed between TB and adenosine. According to the MolDock score, TB has a lower binding affinity than adenosine, as well as other agonists or antagonists. We hypothesize that TB differentially affects protein 3D structure and reduces the efficiency of ARs, thereby disrupting signal transduction and inhibiting GPCR-related activity. However, its effects seem to be lower than those of synthetic drugs (agonists and antagonists). The crystal structure of caffeine in complex with A2AR (3RFM) is known.[46] Caffeine exerts its beneficial effects through A2AR inhibition. Because TB shares the methylxanthine structure with caffeine, it likely inhibits A2AR. Therefore, we removed the caffeine molecule from the binding site in 3RFM and docked TB and adenosine. We found that TB binds better than caffeine. Thus, the observed improvement in ADHD-like symptoms with the long-term ingestion of TB may partially be attributed to the modulation of the dopaminergic nervous system through AR antagonism. Nonetheless, further investigation is warranted to fully understand this complex relationship.

TB supplementation's potential therapeutic mechanism on the central nervous system is hypothesized to function as a nonselective phosphodiesterase (PDE) inhibitor in the brain.[29,63] PDE, which is heterogeneously distributed across various brain regions, including the PFC, when inhibited, leads to an increase in intracellular cAMP levels.[29] This triggers the phosphorylation of CREB and cAMP response element (CRE)-mediated transcription, promoting the production of crucial neural function transcripts such as TH, SNAP-25, and BDNF.[64] In SHR, CREB/CRE-

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dependent transcriptional activity is reduced early in development, potentially leading to decreased transcript expression levels and impaired neurodevelopment in the PFC.[25] Preclinical studies have reported that PDE inhibitors can ameliorate ADHD-like behavior, suggesting that PDEs could be potential targets for pharmacological interventions in neuropsychiatric disorders associated with dopaminergic modulation.[65,66] Consequently, PDE inhibitors are emerging as potential therapeutic candidates for ADHD treatment.[65,66] The potential therapeutic effect of TB as a PDE inhibitor in the treatment of ADHD may require further investigation.

ADHD is a complex disorder influenced by various genetic and environmental factors.[1,2] Accumulating evidence indicates an enhanced inflammatory response as a mechanism for dopamine nervous system dysfunction in both ADHD patients and SHR.[67,68] Epidemiological studies consistently highlight a significant overlap between ADHD and inflammatory/autoimmune disorders. Numerous research has assessed serum inflammatory markers in ADHD patients, while genetic studies have identified polymorphisms in genes associated to inflammatory pathways that may contribute to the disorder.[67-69] In SHR, an increased inflammatory response, particularly during the juvenile stage (around 5 weeks of age), may be linked to disrupted development and maturation of the PFC.[70] Given that TB suppresses inflammatory responses by inhibiting the mammalian target of rapamycin kinase and the nuclear factor-kappa B cascade,[32,71,72] the amelioration of ADHD symptoms in SHR could be attributed to TB's anti-inflammatory effects.

In general, concerns arise regarding the safety of long-term ingestion of pharmaceutical or chemical compound-based therapies. However, this study, involving a 40-day duration of 0.05% TB intake, found no significant changes in liver and renal functions, food intake volume, or body weight in young animals. These findings align with previous studies assessing the functionality and safety of prolonged TB intake.[29,30,32] Notably, TB is generally considered a safer alternative to caffeine, posing a lower risk of causing intoxication.[63] Overall, these results indicate that TB is a safe ingredient for chronic ingestion, although additional safety assessments may be necessary to confirm its efficacy in both juvenile and adult ADHD patients.

Additionally, it's noteworthy that chronic TB ingestion increased BDNF expression in genetic control rats WKY. Previous reports have indicated that chronic TB intake enhances BDNF expression in the PFC, leading to improved motor learning and working memory in rodents,[29,30] consistent with the present results. Consequently, long-term TB ingestion seems to enhance cognition by influencing synaptic plasticity in the PFC, as demonstrated in the ADHD model rats in this study. However, in WKY rats, TB supplementation had minimal impact on dopamine concentration, TH, SNAP-25, dopamine receptor expression levels in the PFC, and locomotor activity. These findings indicate that TB does not excessively stimulate brain function in normal animals, demonstrating its effectiveness in maintaining proper brain function and improving abnormal neurological functions. These observations warrant further exploration of TB effects on

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both healthy subjects and individuals with central nervous system disorders, including ADHD.

This study has several limitations. First, sample size was relatively small for biochemical and histological analyses and variations in the sex and age of test animals were limited. This study would benefit more from a larger and more diverse sample size, with variations in age and sex, to increase the generalizability of the findings to a wider population. Second, although the study included an open-field test and a Y-maze task, additional behavioral assessments and cognitive tests could provide a more comprehensive understanding of the effects of TB on ADHD-like behaviors. Third, it has been hypothesized that dysfunction in various brain regions, including the striatum, substantia nigra, ventral tegmental area, and PFC, is involved in the development of ADHD[1-3]. A comprehensive study including these brain regions may be required to prove the efficacy of TB for ADHD symptoms. Fourth, although SHR is one of the most common animal models for ADHD,[21-25,73] it is controversial.[74,75] Studies using various animal models, such as DAT-knockout mice and neonatal 6-hydroxydopamine-lesioned rats,[74,75] may increase the significance of TB in relieving ADHD symptoms. Fifth, although TB concentration in the diet (0.05% w/w) was determined based on previous studies,[29,30,32] varied doses must be used to determine optimal dosage, i.e., a safer and more effective dose, for alleviating ADHD symptoms.

Conclusions

Our findings represent the initial evidence of the therapeutic potential of TB supplementation to alleviate the behavioral and neurological characteristics associated with ADHD. TB supplementation demonstrated the modulation of dopaminergic neurotransmission factors, including TH, DRD2, DRD4, SNAP-25, and BDNF concentration in the PFC, leading to the subsequent improvement of ADHD-like behavior, i.e., hyperactivity, inattention and working memory deficits, in SHR. The chronic use of TB supplementation holds promise for addressing central dysfunction in ADHD patients, suggesting its potential as a novel lead compound for the treatment of neuropsychiatric diseases.

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Author's contributions

Conceptualization, KM and NS; Formal analysis, KM, SH, RI, and ES; Funding acquisition, KM; Investigation, KM, SH, ES, and NS; Methodology, KM, NS, SH, RI, and HK; Project administration, KM; Resources, KM, NS, MH, HK, and OS; Supervision, NS, MH, HK and OS; Validation, NS and OS; Writing–original draft, KM; Writing–revise and editing, KM and SH. All

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authors reviewed the manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

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Figure legends

Figure 1. Locomotor activity of both the stroke-prone spontaneously hypertensive rat (SHR) and the Wistar–Kyoto rat (WKY). The locomotor activity in SHR was significantly higher compared to WKY. However, the intake of theobromine (TB) notably suppressed this activity in SHR. Results are presented as means ± SE (n = 8 or 10). ###$p < 0.001$ vs. WKY-CN. ***$p < 0.001$ vs. SHR-CN.

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Figure 2. Y-maze task in stroke-prone spontaneously hypertensive rats (SHR) and the Wistar–Kyoto rat (WKY). The parameters measured include (A) the number of total arm entries, (B) the percentage of spontaneous alterations, (C) the alternate arm return (AAR), and (D) the same arm return (SAR) using the Y-maze task. Compared to WKY, SHR exhibited a significantly higher total number of arm entries, which was notably suppressed by theobromine (TB) intake. Additionally, in SHR, the percentage of spontaneous alteration and SAR significantly improved with TB intake. The results are presented as means ± SE (n = 8 or 10). #$p < 0.05$, ##$p < 0.01$ vs. WKY-CN. *$p < 0.05$, **$p < 0.01$ vs. SHR-CN.

Figure 3. (A) Dopamine and tyrosine hydroxylase (TH) concentrations in the prefrontal cortex (PFC) of stroke-prone spontaneously hypertensive rats and the Wistar–Kyoto rats (SHR and WKY, respectively). In SHR, theobromine (TB) ingestion increased dopamine concentrations and normalized TH expression in the PFC. (B) Western blot analysis of TH, dopamine receptor D (DRD) 1−5, dopamine transporter (DAT), and vesicular monoamine transporter-2 (VMAT-2) in the PFC, and (C) densitometric data corresponding to (B). In SHR, the expression of TH, DRD2, and DRD4 was decreased compared to WKY, but this decrease was significantly improved with TB administration. The results are presented as means ± SE (n = 5 or 6). ##$p < 0.01$, ###$p < 0.001$ vs. WKY-CN. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. SHR-CN.

Figure 4. Immunostaining of tyrosine hydroxylase (TH) and neuronal nuclei (NeuN) in the prefrontal cortex (PFC). (A) The presence of TH-positive cells/fibers in the PFC was significantly lower in SHR compared to WKY. However, long-term ingestion of theobromine (TB) notably increased TH expression in the PFC of SHR. Alexa Fluor 488-labeled anti-rabbit IgG and Alexa Fluor 594-labeled anti-mouse IgG were used to detect TH and NeuN immuno-positive cells, respectively. The colors blue, green, and red represent DAPI, TH, and NeuN, respectively. The scale bar represents 50 μm. (B) Quantitative analysis of the mean integral optical density (IOD) of TH in the PFC. Results are presented as means ± SE (n = 3 or 4). ###$p < 0.001$ vs. WKY-CN; ***$p < 0.001$ vs. SHR-CN. (C) Coronal section of a rat PFC. Red square indicates the area presented in (A).

Figure 5. (A) Western blot analysis of synaptosomal-associated protein-25 (SNAP-25), syntaxin 1A, synaptophysin, and synapsin-1 in the prefrontal cortex (PFC) of stroke-prone spontaneously hypertensive rats (SHR) and the Wistar–Kyoto rats (WKY). (B) Densitometric data corresponding to (A). In SHR, the expression of SNAP-25 was decreased compared to WKY, but this decrease was significantly improved with theobromine (TB) administration. Synapsin-1 expression increased by TB supplementation in SHR. The expression levels of synaptophysin and syntaxin were not altered.

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Results are presented as means ± SE (n = 5 or 6). #$p < 0.05$, ##$p < 0.01$ vs. WKY-CN; *$p < 0.05$, **$p < 0.01$ vs. SHR-CN.

Figure 6. Protein expression levels of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex (PFC) of stroke-prone spontaneously hypertensive rats (SHR) and the Wistar–Kyoto rats (WKY). Theobromine (TB) administration increased BDNF concentrations in the PFC in both WKY and SHR. Results are presented as means ± SE (n = 5 or 6). # indicates comparison with control rats (CN). #$p < 0.05$ vs. WKY-CN; **$p < 0.01$ vs. SHR-CN.

Figure 7. Docking to the binding site of the adenosine receptor A2AR with adenosine, caffeine and theobromine (TB). In the binding pocket, (A) adenosine formed hydrogen bonds (blue dashed lines) with Ala63, Ile66, and Ile80. The sterically interacting amino acids (red dashed lines) surrounding adenosine were Ala59, Ala63, Ile66, Ile80, Ala81, Val84, and Ile274. (B) Caffeine did not form hydrogen bonds, whereas (C) TB hydrogen bonded with Ile80. All sterically interacting amino acids in the binding pocket were common (Ala59, Ile66, Ile80, and Val84) between TB and adenosine (Figs A and B, lower panel, represented by crescent). The binding pocket contained a common amino acid, Ile274, which sterically influenced both caffeine and adenosine. Among all ligands, TB docked at a deeper location in the pocket and showed more a negative binding energy score (−77.1 kcal/mol) than caffeine (−65.7 kcal/mol). (Top) 3D structure with a surface model of ligand and protein. (Middle) Ribbon and stick structure of protein and ligand, respectively, visualized by Chimera. (Bottom) Ligand–protein (amino acids of protein) interaction map, developed during docking of ligands onto the adenosine receptor A2AR (PDB ID: 3RFM) by Molegro Virtual Docker (free version).

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Table 1. Body weight (g) in the SHR and WKY control (CN) and theobromine (TB) groups.

	WKY			SHR		
Days	CN (n = 8)	TB (n = 8)		CN (n = 10)	TB (n = 10)	*p*
0	162.4 ± 1.9	169.9 ± 2.3		160.3 ± 1.5	161.9 ± 1.3	0.789
20	211.0 ± 2.3	207.3 ± 1.9		210.3 ± 1.8	214.6 ± 1.2	0.898
40	275.3 ± 2.5	272.5 ± 2.8		270.8 ± 2.5	267.3 ± 1.6	0.632

Values are represented as the means ± SE (n = 8 or 10). We observed no significant differences in body weight between groups CN and TB.

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Table 2. Food intake volume (mg) in the SHR and WKY control (CN) and theobromine (TB) groups.

	WKY			SHR		
Days	CN (n = 8)	TB (n = 8)		CN (n = 10)	TB (n = 10)	*p*
10	17.2 ± 0.2	16.8 ± 0.3		17.6 ± 0.2	17.8 ± 0.6	0.732
20	16.9 ± 0.3	17.7 ± 0.4		17.8 ± 0.4	17.6 ± 0.8	0.812
30	20.1 ± 0.4	21.1 ± 0.6		17.8 ± 0.7	18.0 ± 0.8	0.683
40	22.3 ± 0.6	23.1 ± 1.0		19.9 ± 0.8	19.7 ± 1.0	0.137

Values are represented as the means ± SE (n = 8 or 10). We observed no significant difference in food intake volume between groups CN and TB.

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Table 3. Blood counts in the SHR and WKY control (CN) and theobromine (TB) groups.

□	WKY		SHR		□
□	CN (n = 8)	TB (n = 8)	CN (n = 10)	TB (n = 10)	*p*
WBC ($\times 10^2/\mu L$)	26.2 ±1.6	23.2 ± 2.9	26.0 ± 1.5	25.0 ± 2.7	0.763
RBC ($\times 10^4/\mu L$)	906.8 ± 28.0	925.8 ± 18.5	915.4 ± 8.6	908.5 ± 5.2	0.498
HGB (g/dL)	15.1 ± 0.3	14.8 ± 0.3	15.3 ± 0.2	15.3 ± 0.1	0.571
HTC (%)	44.7 ± 1.0	43.6 ± 1.1	46.4 ± 0.4	46.0 ± 0.3	0.410
MVC (fL)	51.7 ± 1.1	51.8 ± 0.9	50.6 ± 0.1	50.6 ± 0.2	0.901
MCH (pg)	17.1 ± 0.3	17.5 ± 0.4	16.8 ± 0.1	16.8 ± 0.1	0.844
MCHC (g/dL)	33.3 ± 0.4	33.2 ± 0.3	33.1 ± 0.1	33.2 ± 0.2	0.833
PLT ($\times 10^4/\mu L$)	50.9 ± 4.3	54.0 ± 7.5	42.5 ± 4.5	49.8 ± 4.1	0.124

The values are represented as the means ± SEM (n = 8 or 10). WBC, white blood cell; RBC, red blood cell; PLT, platelet; HGB, hemoglobin; HTC, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. There was no significant difference in any blood cell component between the CN and TB groups.

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Table 4. Blood biochemistry in the SHR and WKY control (CN) and theobromine (TB) groups.

□	WKY		□	SHR		□
□	CN (n = 8)	TB (n = 8)	□	CN (n = 10)	TB (n = 10)	*p*
GOT (IU/L)	77.3 ± 4.4	76.6 ± 2.2	□	72.3 ± 4.4	71.6 ± 2.2	0.652
GPT (IU/L)	34.3 ± 1.0	33.7 ± 2.2	□	30.1 ± 1.7	29.7 ± 2.2	0.175
γGT (IU/L)	2.8 ± 0.1	2.9 ± 0.1	□	3.0 ± 0.2	2.9 ± 0.2	0.846
T-Cho (mg/dl)	85.2 ± 3.9	84.6 ± 3.7	□	77.6 ± 4.9	76.6 ± 5.7	0.146
TG (mg/dl)	32.0 ± 8.9	36.6 ± 7.2	□	35.2 ± 6.9	32.6 ± 5.2	0.789
TP (g/dl)	5.2 ± 0.2	5.3 ± 0.1	□	5.4 ± 0.1	5.2 ± 0.1	0.800
Alb (g/dl)	3.2 ± 0.1	3.0 ± 0.1	□	3.1 ± 0.1	3.0 ± 0.1	0.862
BUN (mg/dl)	16.6 ± 0.4	16.3 ± 0.5	□	17.6 ± 0.9	16.8 ± 0.8	0.210
UA (mg/dl)	1.2 ± 0.1	1.0 ± 0.1	□	1.1 ± 0.1	1.2 ± 0.1	0.367
Cre (mg/dl)	0.3 ± 0.0	0.3 ± 0.0	□	0.3 ± 0.0	0.3 ± 0.0	0.966

The values are represented as the means ± SE (n = 8 or 10). We observed no significant difference in any biochemical results between the CN and TB groups. Alb: albumin; BUN: blood urea nitrogen; Cre: creatinine; GOT: glutamate–oxaloacetate transaminase; GPT: glutamate–pyruvate transaminase; γGT: gamma-glutamyl transpeptidase; T-Cho: total cholesterol; TG: triglyceride; TP: total protein.

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Table 5. Comparison of adenosine receptors and ligands affinities

Receptor types	PDB ID	Name of ligand	Score	Positions of amino acids residues	
				Involved in H-bonds	Involved in steric interactions
A1AR	6D9H	Adenosine	−98.5	Tyr12, Glu172, Asn254	Tyr12, Ala66, Asn70, Glu170, Glu172, Asn254
		Theobromine	−66.9	Tyr12, Glu170, Phe171,	Ile69, Phe171, Tyr12, Glu170, Tyr271
		Agonist: N_6 cyclohexyladenosine	−118.9	Phe171, Glu170, Glu 172	Tyr12, Ile69, Asn70, Glu170, Phe171, Glu172, Ile274, Thr277
		Antagonist: Rolofylline	−117.3	-	Ile69, Val87, Thr277
A2AR	2YDO	Adenosine	−110	Tyr88, Ser277, His278, Glu169, Asn253	Thr88, Ser277, His278, Asn253, Glu169
		Theobromine	−64.7	Phe168, Glu169	Phe168, Glu169. Ile66, Ser67, Ile274
		Agonist: Regadenson	−156.9	Asn253, Glu169, Ala63, Tyr9	Glr169. Asn253, Ser67, Tyr9, Ilr274, Ala63, Phe168, Ile66
		Antagonist: Istradefylline	−127.0	Asn253	Asn253, Phe168
A3AR	3EML	Adenosine	−104.3	Ala63, Tyr 271	Tyr9, Ala63, Ile66, Ala81, Ile80, Val84, Phe168, Tyr271
		Theobromine	−71.3	Ile80	Ala59, Ala66, Ile80, Ala81, Val84
		Agonist: IB-MECA	−133.1	Ile66, Tyr271	Ala63, Ile66, Phe168, Tyr271, Ile274
		Antagonist: MRS-1334	−168.6	Tyr271	Phe168, Leu 249, Ile 274, Glu 169, Leu167, Ser 67, Ile66, Tyr 271
A2AR	3RFM	Adenosine	−110.8	Ala63, Ile66, Ile80	Ala59, Ala63, Ile66, Ile80, Ala81, Val84. Ile274
		Caffeine	−65.7	-	Ala59, Ile66, Ile80, Val84. Ile274, His478
		Theobromine	−77.1	Ile80	Ala59, Ala63, Ile66, Ile80, Ala81, Val84

AR, adenosine receptor; PDB, Protein Data Bank archive.

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Figure 1

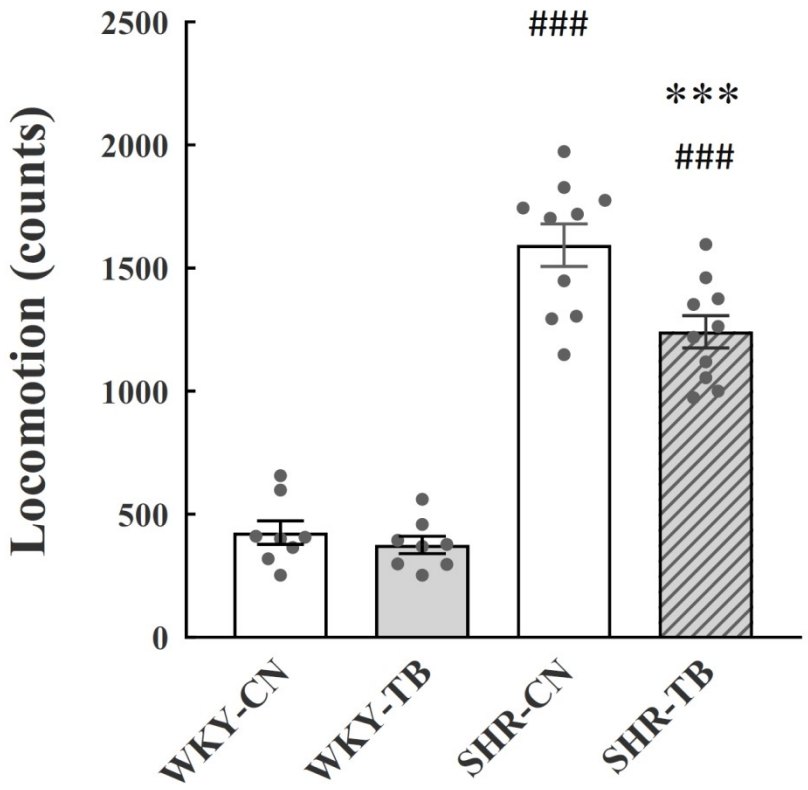

Figure 1

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Figure 2

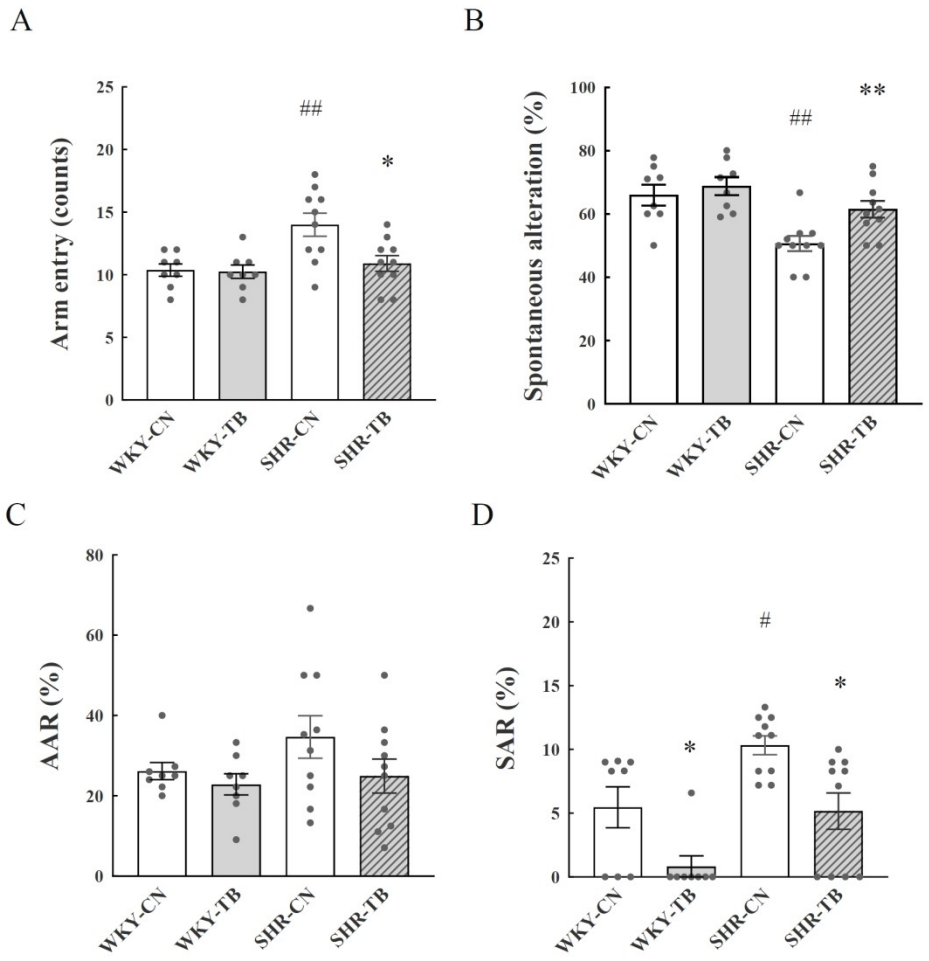

Figure 2

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Figure 3

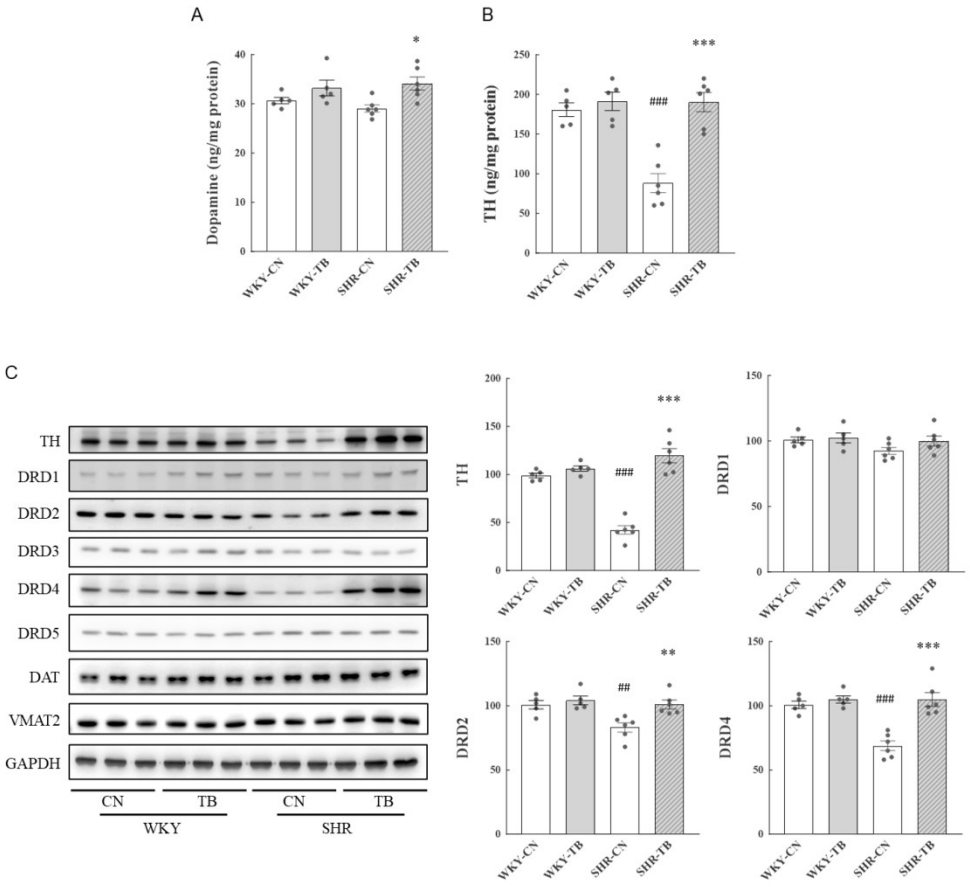

Figure 3

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Figure 4

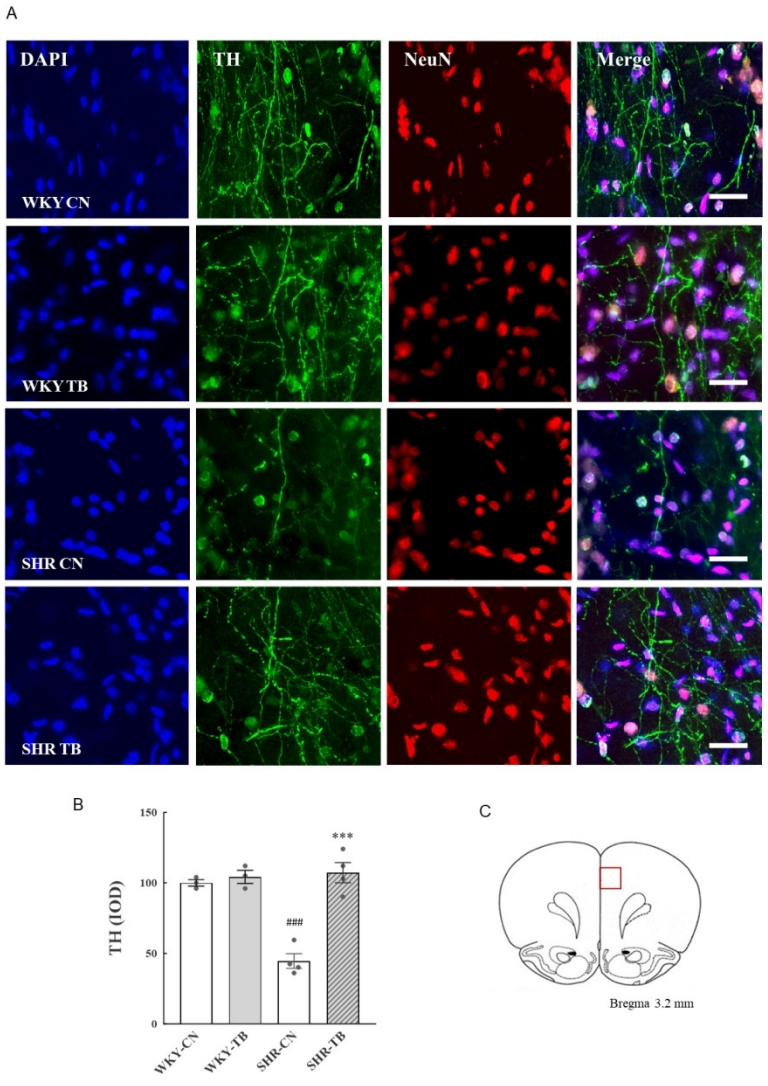

Figure 4

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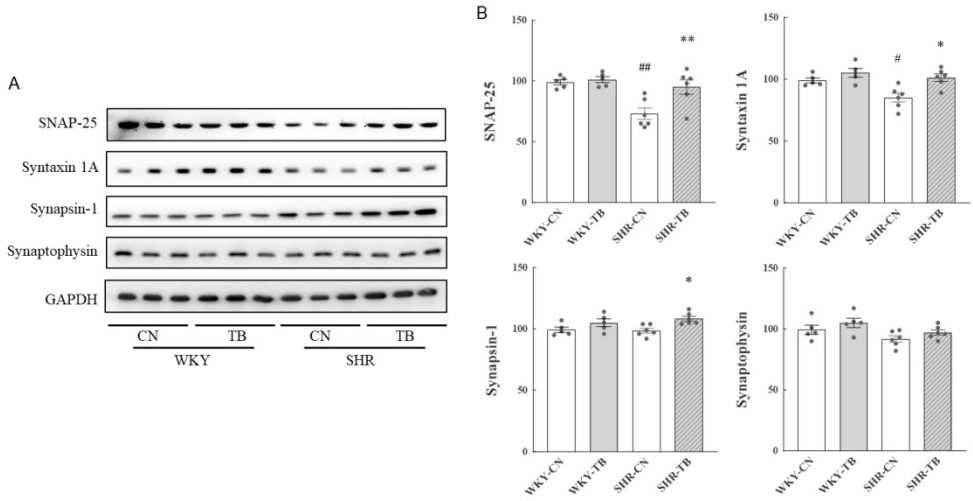

Figure 5

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Figure 6

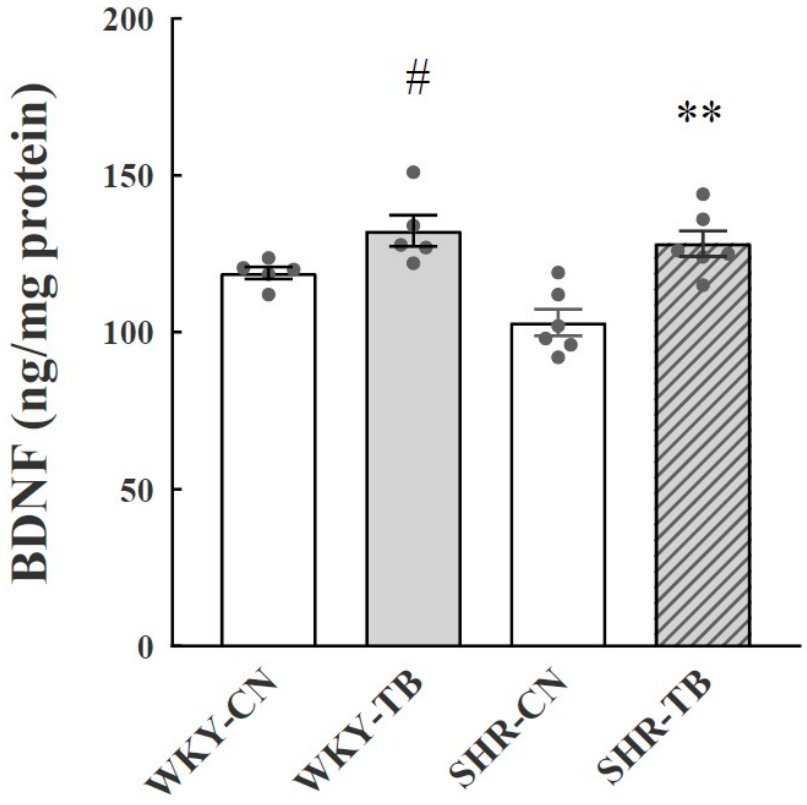

Figure 6

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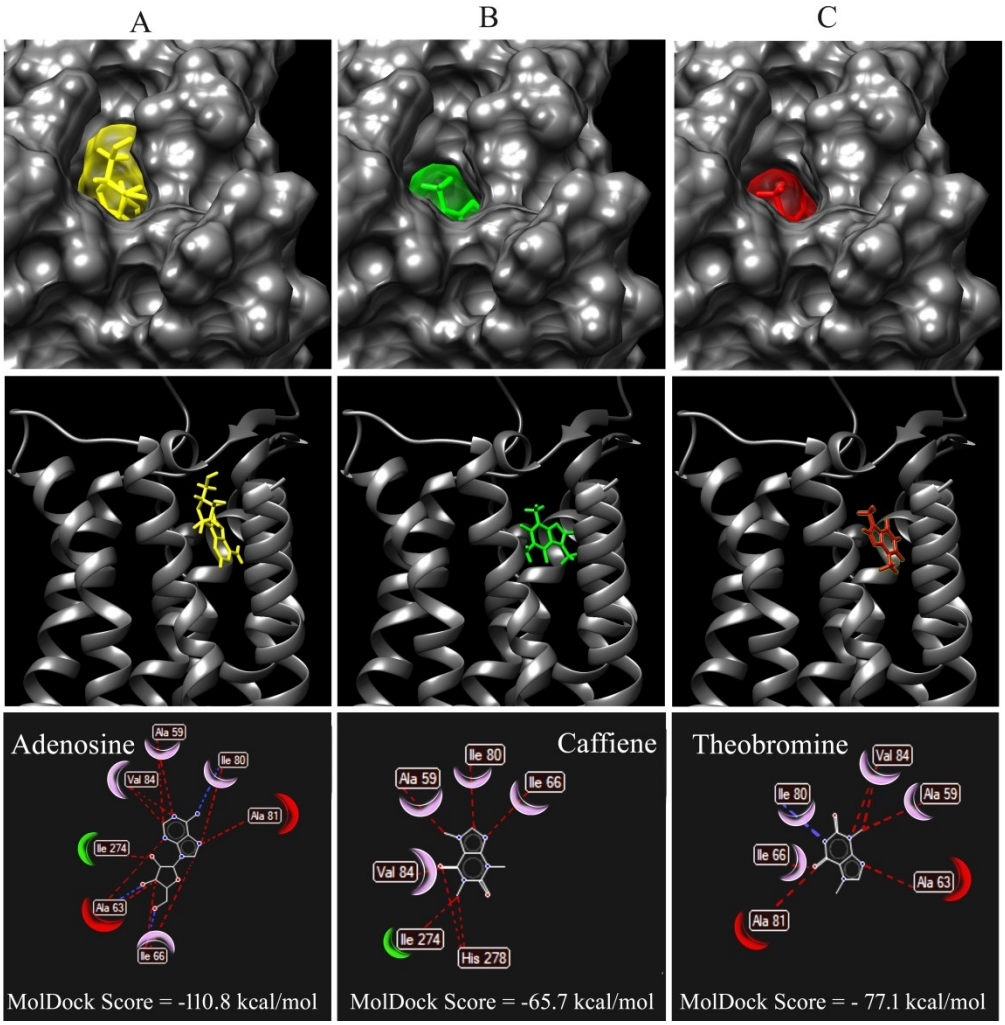

Figure 7

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