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Effects of cordycepin on spontaneous alternation behavior and adenosine receptors expression in hippocampus

Zhi-Ping Cao[a,1], Dan Dai[a,1], Peng-Ju Wei[a], Yuan-Yuan Han[a], Yan-Qing Guan[a], Han-Hang Li[a], Wen-Xiao Liu[a], Peng Xiao[a], Chu-Hua Li[a,b,*]

[a] School of Life Science, South China Normal University, Guangzhou 510631, China
[b] Brain Science Institute, South China Normal University, Guangzhou 510631, China

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ABSTRACT

Cordycepin, an adenosine analogue, has been reported to improve cognitive function. Important roles on learning and memory of adenosine and its receptors, such as adenosine A1 and A2A receptors (A1R and A2AR), also have been shown. Therefore, we assume that the improvement of learning and memory induced by cordycepin is likely related to hippocampal adenosine content and adenosine receptor density. Here we investigated the effects of cordycepin on the short-term spatial memory by using a spontaneous alternation behavior (SAB) test in Y-maze, and then examined hippocampal adenosine content and A1R and A2AR densities. We found that orally administrated cordycepin (at dosages of 5 and 10 mg/kg twice daily for three weeks) significantly increased the percent of relative alternation of mice in SAB but not altered body weight, hippocampus weight and hippocampal adenosine content. Furthermore, cordycepin decreased A2AR density in hippocampal subareas; however, cordycepin only reduced the A1R density in DG but not CA1 or CA3 region. Our results suggest that cordycepin exerts a nootropic role possibly through modulating A2AR density of hippocampus, which further support the concept that it is mostly A2AR rather than A1R to control the adaptive processes of memory performance. These findings would be helpful to provide a new window into the pharmacological properties of cordycepin for cognitive promotion.

1. Introduction

Cordycepin (3′-deoxyadenosine), first isolated from the fermented broth of medicinal mushroom *Cordyceps militaris*, is widely used as a traditional Chinese medicine and exhibits a variety of clinical effects such as immunomodulatory, antioxidant, anti-inflammatory and antimicrobial activities [1–3]. Cordycepin is deaminated quickly by adenosine deaminase and metabolized rapidly to an inactive metabolite, 3′-deoxyhypoxanthinosine in vivo. The half-life and bioavailability of cordycepin by oral administration are 2.1 h and 19.2 μmol h/L (area under concentration-time curve), respectively [4]. In recent studies, cordycepin has been reported to improve cognitive function in mice and protect against cell death induced by cerebral ischemia injury [5,6].

Adenosine is mainly through its action on adenosine A1 receptor (A1R) and adenosine A2A receptor (A2AR) to control and integrate cognition and memory [7]. Adenosine deficiency is able to result in the impairment of synaptic plasticity [8,9]. In the brain, A1R and A2AR are mainly located in synapses, controlling the release of neurotransmitters, such as glutamate (Glu) and acetylcholine (ACh) that are involved in memory and other cognitive processes [10]. Adenosine usually relies on a balance on the activation of inhibitory A1R and facilitatory A2AR [11]. The activation of A1R prevents neuronal damage [12], while the activation of A2AR plays an important effect on the associative learning process and its relevant hippocampal circuits [13]. Previous studies have shown that synaptic levels of adenosine could control synaptic transmission and plasticity by acting on synaptic A1R and A2AR [14,15].

It is worth mentioning that cordycepin, as an adenosine analogue, is structurally similar to adenosine. A previous study has shown that the anti-apoptotic effects of cordycepin are partially dependent on the activation of A1R [16], and cordycepin increases theta waves power density via nonspecific adenosine receptor in rats [17]. These researches indicated that cordycepin may exert potentially biological effects as a nonspecific (or partial) agonist of adenosine receptor. Given that adenosine is mainly through its action on A1R and A2AR to control and integrate cognition functions [7], therefore, we assume that the

* Correspondence author at: School of Life Science, South China Normal University, Guangzhou 510631, China.
E-mail address: lich@scnu.edu.cn (C.-H. Li).
[1] Both authors contributed equally to the paper.

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improvement on learning and memory by cordycepin is likely related to the densities of A1R and A2AR. In order to provide a new window into the pharmacological properties of cordycepin, in the present study, we examined the roles of cordycepin on short-term spatial memory using a spontaneous alternation behavior (SAB) test in Y-maze, and investigated adenosine content and A1R and A2AR densities in hippocampal subregions.

2. Materials and methods

2.1. Drugs and chemicals

Cordycepin (> 98% purity) was obtained by using a column chromatographic method to perform extraction and separation [18]. Adenosine standard and other chemicals were purchased from Sigma Ltd. (Louis, USA). Antibodies of rabbit anti-adenosine receptor A1 (bs-6649R) and rabbit anti-adenosine receptor A2A (bs-1456R), and SP kit (rabbit, SP-0023) were bought from Bioss Company (Beijing, China). The constitution of the secondary kit is biotin-labeled goat anti-rabbit IgG.

2.2. Animals, ethics statements and groups

All studies were reported in accordance with the ARRIVE guidelines for all the experiments involving animals. Animal procedures undertaken were approved by the Committee on Animal Care and Usage of South China Normal University and every effort was made to minimize animal suffering. Kunming mice (an outbred mouse stock deriving from Swiss albino mice with a high ratio of gene heterozygosis, 25–28 g, 6 weeks of age) were obtained from the animal facility at the Sun Yat-sen University (Guangzhou, China) [19]. The animals were housed in the animal facility of South China Normal University in standard conditions. Food and water were supplied ad libitum. Room temperature was 23–25 °C, relative humidity was 40–50%, and the day/night cycle was set at 12 h/12 h. All animals were acclimatized for 3 days before starting any procedures. Mice were group housed (6 mice per cage) in conventional laboratory rodent cages (ZS Dichuang Co., Beijing, China) of dimensions 18.7 (W) × 15.5 (H) × 29.5 (L) cm. Animals were selected randomly from all cages and divided into groups. All analyses were carried out without prior knowledge of the treatments. Animals received a numerical code throughout the whole experiment.

The current study included two experiments: a preliminary experiment and a formal experiment. In the preliminary experiment, mice were divided into male (n = 31) and female (n = 28) groups and tested to obtain an appropriate detection time period. According to the results of the preliminary experiment, 84 male mice were randomly divided into three groups in the formal experiment: control, 5 and 10 mg/kg (0.1 mL/10 g body weight) cordycepin groups. Cordycepin was administered intragastrically twice daily (in the morning and afternoon) for 3 weeks. Animals in the control group were treated with an equal volume of double distilled water. Mice were evaluated using the SAB test in a Y-maze at least 24 h after the final drug treatments.

2.3. SAB test

As described previously [20], SAB, a hippocampus-dependent behavior test to assess attention toward novelty and spatial memory, was performed in a Y-maze with identical dimensions (45 cm long × 14 cm wide × 16 cm high with arms) at a 120° angle from each other. Each mouse was placed at the Y-maze center and allowed to move freely for 8 min (30 min in the preliminary experiment). Over the course of multiple arm entries, the animal typically shows a tendency to enter a less recently visited arm. The numbers of arm entries and alternation (triads) were recorded, respectively. An entry occurred when all four limbs were within the arm. Alternation was defined if mouse's arm entry was different from the two previously entered arms; an error was recorded if the mouse went back to either of the two arms just previously visited. The percentage of relative alternation during a period of 5 min (from 3 to 8 min) observation was calculated from the ratio of the number of alternations divided by the number of total entries [21,22]. The value was multiplied by 100. Experiments and statistical analysis were double blinded.

At the end of behavioral test, mice were randomly divided into two parts: HPLC and immunohistochemistry. All mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then carried out the next experiments.

2.4. HPLC procedure

Anesthetized mice were sacrificed by rapid decapitation. The brains were removed and carefully rinsed in ice-cold saline to remove any blood contaminants. Bilateral hippocampus was dissected quickly to ensure bioactivity of tissues. After weigh and homogenization in 2.0 mL Eppendorf tubes, the sample was thawed and minced in 0.4 mmol/L HClO4 (200 μL) for 2 min. The homogenate was centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was transferred and filtered through a 0.22 μm pore size Milipore filter for HPLC analysis.

Analysis was carried out by using Waters-HPLC system (Waters, Massachusetts, USA) in 240 nm. The sample was separated with an XTerra MS C18 column (5 μm, 4.6 × 250 mm column, USA) manufactured by water. The mobile phase was composed of a mixture of acetonitrile-water solution (0.6 mL/min, 5: 95, v/v) filtered through a 0.45 μm nylon filter and degassed under ultrasound and vacuum for 30 min, with injecting 10 μL solution into the equilibrated HPLC system every time. Adenosine standard was dissolved in distilled water in concentrations of 2, 4, 6, 8, 10 and 20 μg/mL and then established a standard curve. The content of adenosine in hippocampus was calculated by dividing hippocampal weight.

2.5. Immunohistochemistry

Briefly, mice were anesthetized followed by perfusion with 0.9% NaCl and then 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4. The brains were removed and post fixed for 1 h at 4 °C refrigerator, and then stored in 30% sucrose in phosphate buffer for cryoprotection. Ceyostat sections in 30 μm thickness were received and stored at 4 °C in 0.01 M PBS and transferred to sodium citrate for microwave repair (medium heat, 10 min for 2 times). After washed by 0.01 M PBS for 2 times and treated with 3% H_20_2 for 20 min to eliminate influence of endogenous peroxidase, monoclonal antibodies of A1R and A2AR were used at a dilution of 1: 200 at 4 °C refrigerator for 24 h and then placed in 37 °C incubator for 30 min. Washing by 0.01 M PBS and secondary antibodies treatment, sections were processed with horseradish peroxidase-biotin-avidin complex for 20 min by DAB for 5 min, and then ended the reaction with 0.01 M PBS for 3 times. Sections finishing gradient ethanol dehydration were mounted on gelatinated slides and plated on coverslips.

Hippocampal CA1, CA3 and DG areas were photographed under 20 × objectives with light microscopy. Image-pro Plus Microsoft (Media Cybernetics, USA) was used for optical density analysis.

2.6. Statistical analysis

All data were expressed as mean ± SEM and then analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Origin 7.5 software (Northampton, MA, USA) and SPSS 17.0 package (Chicago, IL, USA) were employed for graphing and data analysis. The level of statistical significance was set at $p < 0.05$.

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