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Research article

Protective effect of CD73 inhibitor α, β-methylene ADP against amyloid-β-induced cognitive impairment by inhibiting adenosine production in hippocampus

Wu Song, Yong Tang, Lin Wei, Chi Zhang, Danning Song, Xueting Li, Shuang Jiang *

Department of Pharmacology, School of Medicine, Changchun University of Chinese Medicine, No.1035 Boshuo Road, Changchun 130117, China

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ABSTRACT

Background: Alzheimer's disease (AD) is a chronic, progressive neurodegenerative disease. Recent studies have reported the close association between cognitive function in AD and purinergic receptors in the central nervous system. In the current study, we investigated the effect of CD73 inhibitor α, β-methylene ADP (APCP) on cognitive impairment of AD in mice, and to explore the potential underlying mechanisms.
Results: We found that acute administration of $A\beta_{1-42}$ (i.c.v.) resulted in a significant increase in adenosine release by using microdialysis study. Chronic administration of APCP (10, 30 mg/kg) for 20 d obviously mitigated the spatial working memory impairment of $A\beta_{1-42}$-treated mice in both Morris water maze (MWM) test and Y-maze test. In addition, the extracellular adenosine production in the hippocampus was inhibited by APCP in Aβ-treated mice. Further analyses indicated expression of acetyltransferase (ChAT) in hippocampus of mice of was significantly reduced, while acetylcholinesterase (AChE) expression increased, which compared to model group. We observed that APCP did not significantly alter the NLRP3 inflammasome activity in hippocampus, indicating that anti-central inflammation seems not to be involved in APCP effect.
Conclusions: In conclusion, we report for the first time that inhibition of CD73 by APCP was able to protect against memory loss induced by $A\beta_{1-42}$ in mice, which may be due to the decrease of CD73-driven adenosine production in hippocampus. Enhancement of central cholinergic function of the central nervous system may also be involved in the effects of APCP.
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1. Introduction

Alzheimer's disease (AD), an irreversible neurodegenerative disease with clinical characteristics of memory loss and cognitive impairment, has been a serious social and health problem worldwide. Despite decades of research, the exact mechanism of AD is still elusive. At present, ATP and its metabolite adenosine, as neurotransmitters in the central nervous system (CNS) have shown profound neuromodulatory effects and have drawn high research interest [1,2,3,4]. With the deepening of the research on purine receptors, researchers have proposed the theory of "purine signaling", including the P1 (adenosine receptor, including A1, A2A, A2B and A3) and P2 receptors (including $P2X_{1-7}$ and $P2Y_{1,\ 2,\ 4,\ 6,\ 8,\ 11-14}$). Adenosine receptors (A-R) are activated by adenosine, while P2 receptors are activated by extracellular nucleotides including ATP [5].

Accumulating evidence reveals that ATP and adenosine in the CNS play important roles in the pathogenesis of AD [5,6,7,8,9]. There is now a widespread consensus that the pathogenesis of AD is accompanied by the enhancement of adenosine receptor-mediated excitatory damage leading to impairing learning and memory [5,10]. It is noteworthy that many epidemiological investigations have shown that people who have the habit of drinking coffee are less likely to develop AD, which is due to antagonizing central A-R by caffeine [11,12]. Intriguingly, in contrast to the adenosine receptors, activation of ATP receptors shows protective effect on AD [13]. Given the opposite role of the adenosine and P2 receptors in the pathogenesis of AD, it is therefore important to regulate the balance of endogenous ligands of the two receptors signaling. Extracellular adenosine is

* Corresponding author.
E-mail address: jiangshuang_2000@163.com (S. Jiang).
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mainly formed from AMP by ecto-5-nucleotidase (CD73), a glycoprotein anchored on the cell membrane, which catalyzes the metabolism of nucleotides to nucleoside [14]. Therefore, intervention of CD73 may regulate the content of extracellular ATP and adenosine, and thus affect both P1 and P2 receptors. However, up to date, no data are yet available about CD73 and AD. We hypothesized inhibition of CD73 activity might be benefit to AD.

Accordingly, in the current study, we investigated whether the CD73 inhibitor α, β-methylene ADP (APCP) was able to protect $A\beta_{1-42}$-induced learning and memory deficit by decreasing CD73-derived adenosine in hippocampus of mice. In addition, brain microdialysis and high-performance liquid chromatography (HPLC) were used to observe the effects of APCP on the changes of extracellular adenenosine levels in the hippocampus of mice. To further investigate the underlying mechanism, nod-like receptor protein-3 (NLRP3) inflammasome and cholinergic function in CNS are also evaluated.

2. Materials and methods

2.1. Drugs, reagents and equipment

Alpha, beta-methylene-adenosine-5′-diphosphate (APCP, CAS:768-14-7, B21045) was purchased from Sigma (USA); interleukin-1 (IL-1) assay Kit (H002), interleukin-18 (IL-18) assay Kit (H015), acetylcholinesterase (AChE) assay kit (A024-1-1) and choline acetyltransferase (CHAT) assay kit (A079-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-NLRP3 (D4D8T) rabbit monoclonal antibody (catalog No. 15101, 1:200 dilution), anti-cleaved caspase-1 (Asp296, E2G2I) Rabbit monoclonal antibody (catalog No. 89332, 1:500 dilution) and anti-rabbit IgG HRP-linked antibody (catalog No. 89332) were from Cell Signaling Technology (USA). Anti-acetylcholinesterase rabbit monoclonal antibody (catalog No. ab183591, 1:500 dilution) and anti-choline acetyltransferase rabbit monoclonal antibody (catalog No. ab178850, 1:200 dilution) were from Abcam (Cambridge. UK). BCA protein assay kit (P0012S), Bovine albumin (ST023) and Glycine (ST085) were purchased from Beyotime Biotechnology (Beijing, China). Adenosine (CAS:58-61-7, A9251) and donepezil hydrochloride (CAS:120011–70-3, D6821) were from Sigma-Aldrich (St. Louis, MO, USA). The stereotaxic apparatus (68025) and syringe pump (KDS Legato 130) were purchased from RWD Life Science (Shenzhen, China). Other reagents and drugs were purchased commercially. Waters 2795 HPLC system coupled to a Waters 2487 were employed for adenosine content detection. The polyvinylidene difluoride (PVDF, IPVH00010) membrane was purchased from Merck Millipore (Billerica, MA, USA). The Y-maze (BW-MYM103) and Morris water maze (YH-MWM) equipment were purchased from Shanghai Bio-will Co., Ltd. (Shanghai, China).

2.2. Animals

Male C57BL/6J mice weighing 18–22 g provided by Jilin University Laboratory Animal Center [animal license No.: SCXK (2011-0004)] were raised under SPF conditions. The animal experiments were carried out in consistent with the provisions of China Animal Welfare Act and the Guide of NIH Experimental Animal Management and Use after being approved by the Ethical Committee of Experimental Animals of Changchun University of Traditional Chinese Medicine.

2.3. Experimental designs

To ascertain the changes of adenosine release and CD73 expression in AD, the brain-microdialysis and western blot were applied to examine the effect of acute $A\beta_{1-42}$ injection on extracellular adenosine content and CD73 protein expression. Twenty-two mice were randomly divided into 2 groups. Eight mice were used for microdialysis study and three mice for CD73 protein detection by western blot.

Next, in the acute brain microdialysis experiment, 18 mice were randomly divided into 3 groups, including control group, APCP 10 mg/kg group and APCP 30 mg/kg group. After adaptive breeding for 7 d, mice were subjected to microdialysis operation. Next day, mice in each group were injected with APCP (10 and 30 mg/kg) or saline. The dialysate samples were collocted every 20 min and the changes of hippocampal adenosine levels were measured within 180 min by using high-performance liquid chromatography (HPLC) with UV detection.

Seventy-two mice were randomly divided into 6 groups ($n = 12$/group), including control group, sham-operated (S-O) group, model group, APCP 10 mg/kg group, APCP 30 mg/kg group and donepezil group. Mice were anesthetized with chloral hydrate (320 mg/kg, i.p.), $A\beta_{1-42}$ (1.5 nmol/5 μl each) or artificial cerebrospinal fluid (ACSF, for the sham operation group) was then slowly injected into the lateral ventricle within 3 min. The components of ACSF contain NaCl (147 mM), $CaCl_2$ (2.2 mM), KCl (4 mM) and KH_2PO_4 (1.2 mM). From the second day, the APCP 10 and 30 mg/kg group received intraperitoneal injection of APCP (10 and 30 mg/kg) daily for 20 consecutive days. The control group and model group were injected with saline (0.1 ml/10 g) once daily. The donepezil (DPZ) group was injected with DPZ (0.6 mg/kg, i.p.). Locomotion activity was measured on the 7th day after $A\beta_{1-42}$ injection; Y-maze were measured on the 8th d and Morris water maze experiment was performed on the 14th–19th d. Six hippocampal tissues homogenates from each group were used for microdialysis study, while other six hippocampal tissues in the same group were used for Elisa and western-blot assay. The experiment schedule is shown in Fig. 1.

2.4. Y-maze test

The Y-maze consists of three arms with an included angle of 120°. The size of each arm is 30 cm * 8 cm * 15 cm (length * width * height). Mice were initially placed in one arm, and the order and number of each mouse was manually recorded over a period of 8 min. After the measurement of each mice, 75% alcohol was used to eliminate the taste in the arm of the Y maze. Alternation rate% = number of alternations / (total number of times - 2)%.

2.5. Locomotor activity test

The locomotor activity test and the microdialysis experiment were simultaneously performed. The size of locomotor activity box for mice

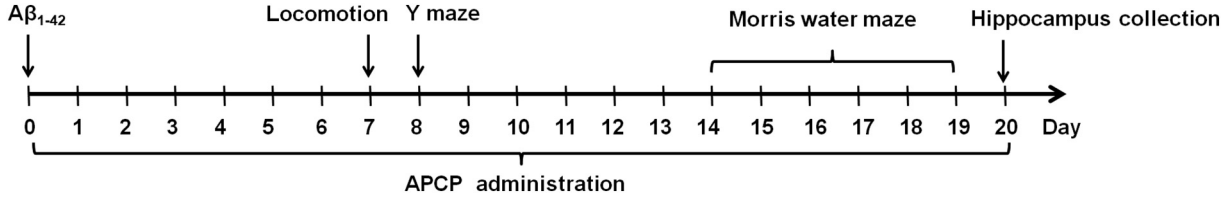

Fig. 1. Schematic representation of experimental procedure.

was 30 cm × 30 cm × 45 cm, the inside of the box was black, and a camera and infrared light source were equipped with at the top of box. After the beginning of experiment, the mice were placed in the box, locomotor activities of the mice were recorded after they moved freely for 10 min, and the recording lasted 30 min.

2.6. Morris water maze test

The Morris Water Maze Test was performed 1 h after the end of the administration on the 14th day. The Morris water maze is a barrel-shaped iron device with a bottom surface diameter of 80 cm and a height of 30 cm. The top surface is open. The bottom surface is marked with quadrants I, II, III, and IV, respectively, and a platform is placed at quadrant III. The platform is located 1 cm below the water surface. During the experiment, the time from entering the water to finding the platform and successfully climbing the platform was recorded as the incubation period. If the mice failed to climb the platform within 90 s, the latency was 90 s. Each mouse swims twice a day, at intervals of 3 h, for 5 consecutive days. After the 5 d training, the platform was evacuated and a probe test was performed to record the swimming time, number of times of crossing the platform within 90 s.

2.7. Brain microdialysis and adenosine measurement

The microdialysis method with a little modification was used to analyze adenosine from previous research [15,16]. Briefly, the experimental mice were anesthetized with chloral hydrate, fixed to a stereotactic brain, and the skull was exposed. A dialysis probe was implanted laterally into the hippocampal region of the brain (coordinates: A -2.2 mm from bregma, V -2.5 mm from occipital bone). This kind of probe could collect the neurochemicals of interest from both sides of the hippcampus. The mice were subjected to microdialysis experiments 24 h after waking. The dialysis probe is connected to an automatic perfusion pump on one side and an EP tube (200 μl) on the other. The perfusion rate was 4 μl/min. Discard the first 2 h of dialysate and collect samples every 20 min. Ensure that the basic value determination of three consecutive samples has an error of <5%.

The levels of microdialysate adenosine were quantified by HPLC with UV detection. The HPLC method for detecting adenosine is consistent with our previous research [15]. Briefly, liquid phase conditions: Column C18 (4.6 μm × 250 mm); mobile phase 0.02 mol/l NaH_2PO_4, 2% acetonitrile, pH adjusted to 4; wavelength 260 nm; flow rate 1 ml/min.

2.8. Western-blot assay

The hippocampal protein samples were extracted with RIPA buffer containing phosphatase inhibitors. 8–10% SDS-PAGE was employed with 20 μg sample volume and 80/120 V constant voltage, which followed by transferring onto polyvinylidene difluoride (PVDF) membranes (0.45 μm). The membrane was blocked by 5% BSA for 1 h under 37°C. After washing 3 times, the membrane was incubated with murine internal reference protein β-actin antibody (1:1000), cleaved caspase-1 (1:500), NLRP3 (1:500), AChE (1:500) and CHAT (1:500) in TBST containing 5% BSA. Subsequently, the membranes were incubated with the horseradish peroxidase-conjugated IgG as the secondary antibody for 1 h. After the PVDF membrane was washed with TBST buffer, ECL reagent was added for fluorescence. The relative protein expression was expressed as the ratio to β-actin. The density of staining was calculated using Image-Pro Plus 6.0.

2.9. Evaluation of ACHE, CHAT and inflammatory cytokines in hippocampus

The levels of IL-1β and IL-18 content, as well as AChE, CHAT activities were determined by Elisa kit according to the manufacturer's protocol.

2.10. Statistical analysis

Statistical analysis was carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The data in this article are expressed in mean ± SEM. Statistical comparisons of data between the two groups were performed by using student's *t* test (normal distribution, Fig. 2B) or Mann–Whitney U-test (abnormal distribution, n > 4, Fig. 2A). To compare the differences among three or more groups, normally distributed continuous variable data was analyzed using one-way ANOVA with *post-hoc* Tukey 's HSD test or Dunnett 's t test for comparison between each group, while non-normally distributed data was used Kruskal-Wallis H test, followed by Nemenyi test or Mann–Whitney U-test for comparison between individual two group. The statistical difference is set at $P < 0.05$.

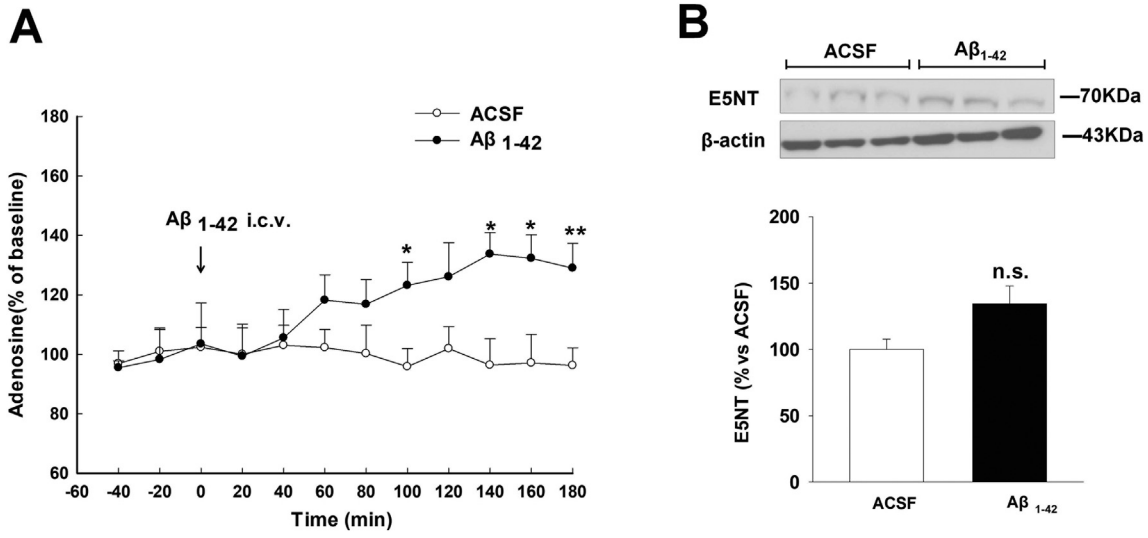

Fig. 2. Effect of acute administration of $A\beta_{1-42}$ on adenosine release (A) and CD73 protein expression in hippocampus of mice (B). $A\beta_{1-42}$ (4 μl, 1 μg/μl) was acute administrated (i.c.v.). For microdialysis study, each point represents the percentage changes from basal values. The basal levels were considered as the mean of substance concentrations in the three samples before drug administration. The hippocampus tissues were collected after brain microdialysis procedure. The relative optical densities of CD73 normalized to β-actin are shown below the bands. All data are expressed as means ± SEM ($n = 8$/group for microdialysis experiment, $n = 3$/group for western blot). For statistical significance, $^*P < 0.05$, $^{**}P < 0.01$ compared with the ACSF group. N.s. represents no significant difference between groups.

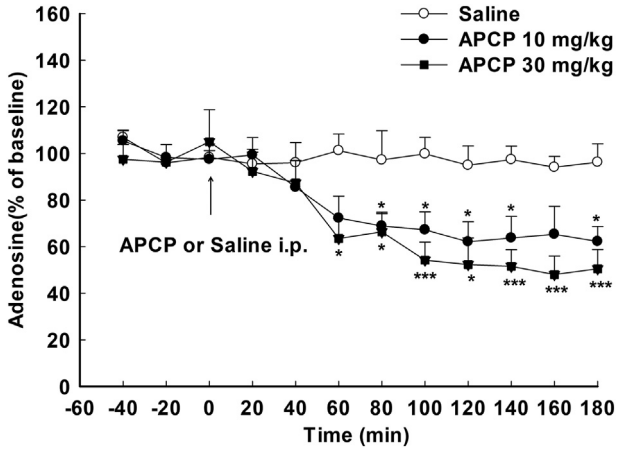

Fig. 3. Effect of acute administration of CD73 inhibitor APCP on hippocampal adenosine release of mice. APCP (10 and 30 mg/kg) was acute administrated (i.p.). Each point represents the percentage changes from basal values. All data are expressed as means ± SEM (n = 6–8/group). For statistical significance, $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the saline group.

3. Results

3.1. Aβ$_{1-42}$ promotes adenosine release and CD73 expression in hippocampus

In order to investigate whether CD73 is involved in Alzheimer's disease, the brain-microdialysis was applied to examine the effect of acute Aβ$_{1-42}$ on extracellular adenosine content and protein expression of CD73 in hippocampus. As shown in Fig. 2A, Aβ$_{1-42}$ (4 μl, 1 μg/μl, i.c.v.) administration significantly evoked the release of adenosine in the hippocampus to 133.46 ± 7.19% of baseline at 140 min ($P < 0.05$). As shown in Fig. 2B, The Western blot results showed that the acute administration of Aβ$_{1-42}$ led to a slight increase of CD73 expression in the hippocampus, but not reached statistical significance. These data showed that acute administration of Aβ$_{1-42}$ resulted in the release of adenosine in hippocampus. The changes in CD73 expression seem to be involved, maybe partly, in the Aβ$_{1-42}$-mediated adenosine release. This result suggests that the CD73-dependent extracellular adenosine release may be involved in the cognitive impairment caused by Aβ$_{1-42}$, prompting us to further explore the changes of adenosine content and CD73 in AD model.

3.2. CD73 inhibitor APCP decreases adenosine release in hippocampus

Next, we examined whether inhibition of CD73 could reduce the release of extracellular adenosine in hippocampus. The α, β-methylene ADP (APCP), a CD73 inhibitor, was employed. As shown in Fig. 3, compared to saline group, acute administration of APCP (10 and 30 mg/kg, i.p.) produced a maximal decrease to 61.79 ± 8.22% (APCP, 10 mg/kg) and 45.13 ± 5.30% (APCP, 30 mg/kg) of baseline 120 min and 160 min after the beginning of administration ($P < 0.05$ and $P <$ 0. 001, for APCP 10 or 30 mg/kg, respectively).

3.3. APCP attenuates the impairment of recognition in Y-maze test

In order to investigate the effect of APCP on cognitive deficits caused by Aβ$_{1-42}$, the Aβ$_{1-42}$-induced mouse AD model was established. Eight days after Aβ$_{1-42}$ administration, the effect of APCP on working memory was carried out in the Y-maze test. As shown in Fig. 4A, the

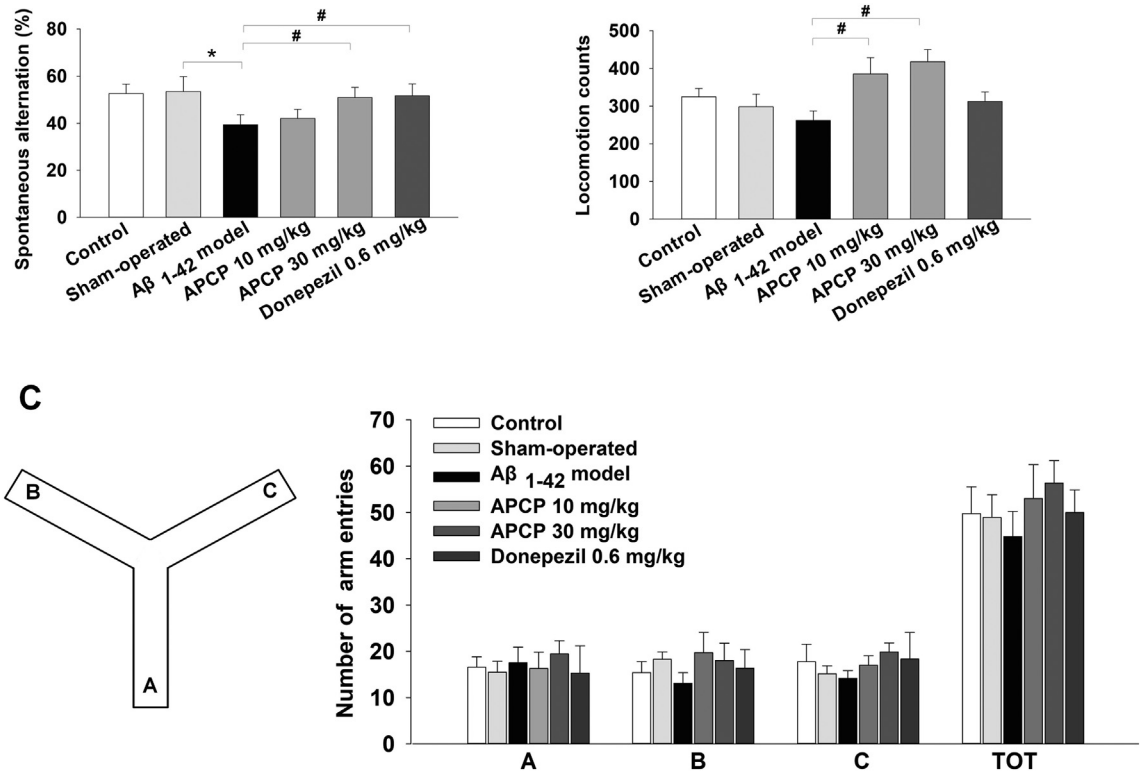

Fig. 4. Effect of APCP on Aβ$_{1-42}$-induced memory deficits in the Y-maze test. The Y-maze test, including spontaneous alternation (A) and number of each arm entries (C) during an 8 min session was performed 8 d after Aβ$_{1-42}$ injection, and the locomotion activity (B) in 1 h was measured 7 d after Aβ$_{1-42}$ injection. APCP (10 and 30 mg/kg, i.p.) was administrated for 20 d. The donepezil positive control group was treated with donepezil (0.6 mg/kg, i.p.). All data are expressed as means ± SEM (n = 11–12/group). For statistical significance, $^{*}P < 0.05$ compared with the sham-operated (S-O) group; $^{\#}P < 0.05$ compared with Aβ$_{1-42}$ model group.

spontaneous alternation in $A\beta_{1-42}$ group was significantly lower than that in S-O group by 13.11 ± 2.08% ($P < 0.05$). APCP (30 mg/kg) significantly reversed the lowered spontaneous alternation induced by $A\beta_{1-42}$ ($P < 0.05$). On the 7th day, locomotion activity of the mice was measured by mice activity recorder (Fig. 4B). It is unexpected that APCP (both 10 and 30 mg/kg) significantly increased the excitability of mice compared to S-O group ($P < 0.05$). In order to avoid the experimental results of the spontaneous alternation being affected by the excitability caused by APCP, we then detected the numbers of each arm entries. As shown in Fig. 4C, there was no significant alteration of numbers of arm entries in all experimental groups.

3.4. APCP attenuates the impairment of recognition Morris water maze

To assess the spatial learning and memory ability, the Morris water maze test was employed. Fig. 5A shows the swimming paths of each group on the second and fifth days of the process. As shown in Fig. 5B, the time spent to find a platform has gradually decreased in all groups. However, the latency of finding a platform for each group was significantly different. Compared with S-O group, the $A\beta_{1-42}$ caused mice to spend significantly more time finding a platform from the third day ($P < 0.05$). From day 4 to day 5, the extended escape latency was significantly shortened by APCP (both

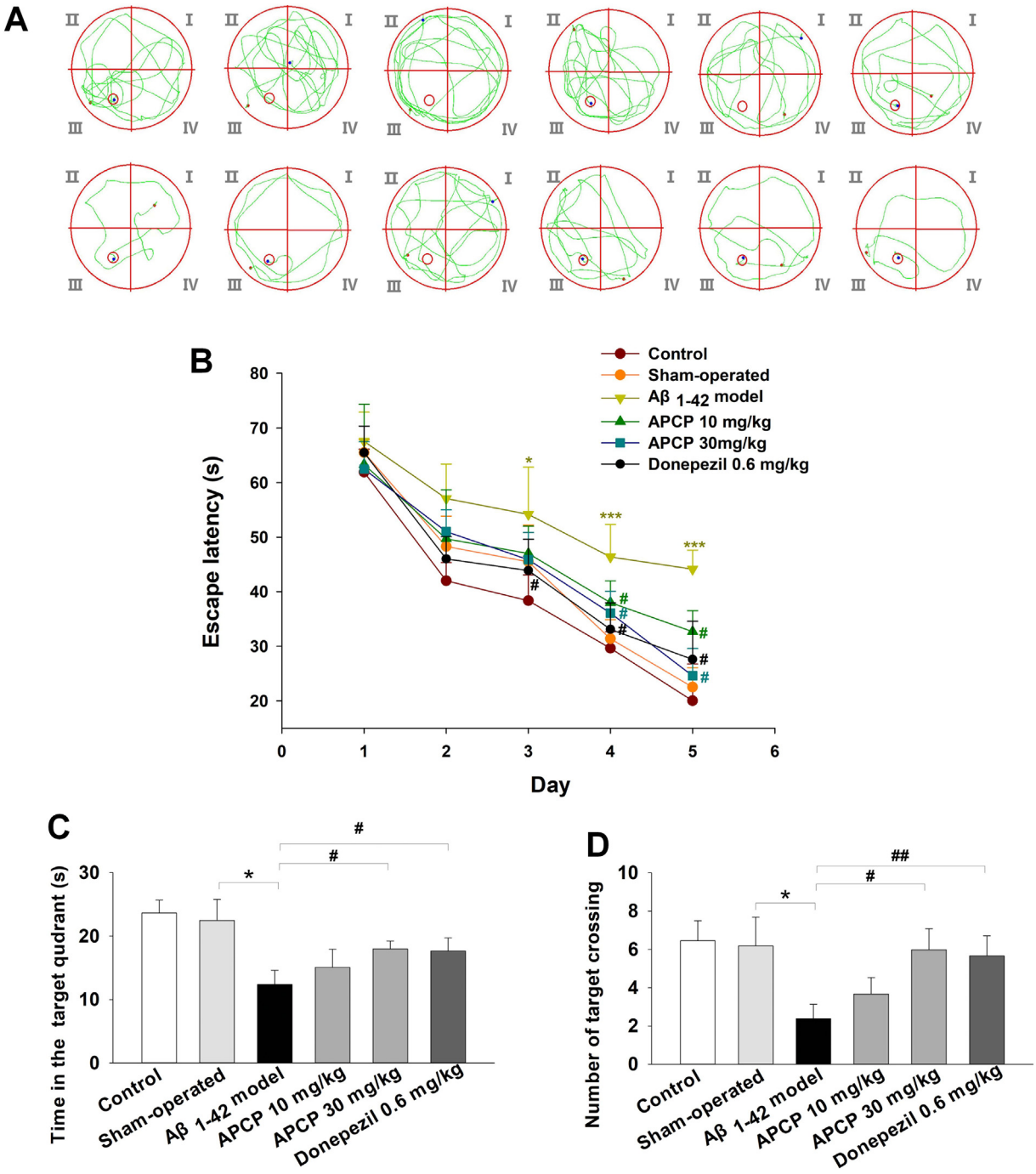

Fig. 5. Effect of APCP on the performance in Morris water maze of $A\beta_{1-42}$-induced memory deficits mice. (A). Search strategy of mice in the second trial on the second (first row) and fifth day (second row). (B). Escape latency to find the hidden platform during the 5-d training. (C). The time spent in the quadrant where the platform located. (D) Numbers of target quadrant crossing. The Morris water maze test was performed 14 d after $A\beta_{1-42}$ injection for 5 consecutive days. All data are expressed as means ± SEM (n = 10–12/group). For statistical significance, $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the sham-operated (S-O) group; $^{\#}P < 0.05$ compared with $A\beta_{1-42}$ model group.

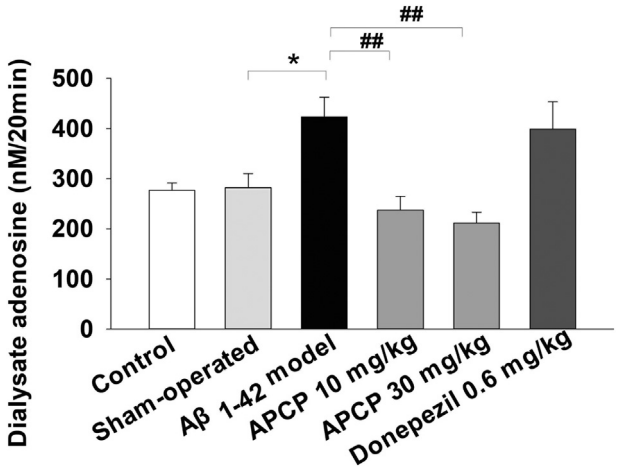

Fig. 6. Effect of APCP on adenosine levels in the dialysate in hippocampus of $A\beta_{1-42}$-induced memory deficits mice. The microdialysis experiment was carried out after all behavior study. After about 2 h, the dialysis samples were collected during 60 min. All data are expressed as means ± SEM ($n = 5–6$/group). For statistical significance, $^{*}P < 0.05$ compared with the saline group; $^{\#}P < 0.05$, $^{***}P < 0.001$ compared with $A\beta_{1-42}$ model group.

10 and 30 mg/kg). Compared with APCP group, donepezil took effect more rapidly (on day 3), but the escape latency showed not significantly different from the APCP group. In the probe test, the mice in the $A\beta_{1-42}$ group were significantly reduced to almost half of the S-O group in both the time of the quadrant and the numbers of crossing (Fig. 5C and D). The learning and memory impairment could be significantly reversed by APCP 30 mg/kg almost to the control group. These data indicate that APCP could significantly improve the learning and memory impairment of mice induced by $A\beta_{1-42}$ in the Morris water maze.

3.5. APCP decreases the extracellular concentration of adenosine in Aβ1–42-treated mice

The effect of APCP on hippocampal adenosine levels in the dialysate were carried out by microdialysis. As shown in Fig. 6, the results showed that, compared with the blank group, acute administration of Aβ1–42 significantly increased the adenosine content in the hippocampal dialysate to 405.27 ± 37.83 nM/20 min. This effect was inhibited by APCP (10 and 30 mg/kg, $P < 0.05$), while donepezil did not show this effect ($P > 0.05$).

3.6. APCP shows no effect on NLRP3 inflammasome activation

As shown in Fig. 7A and B, the results showed that the content of IL-1β in the hippocampus of the model group is significantly increased, and there is no significant difference in the IL-18 of each group. APCP could increase the level of IL-1β, without significant effect on IL-18 content ($P > 0.05$). As shown in Fig. 7C, the western blot results showed that compared with the S-O group, the protein expression of NLRP3 and cleaved Caspase-1 in the model group was significantly up-regulated ($P < 0.05$). However, long-term administration of APCP had no significant effect on the up-regulation of NLRP3 and cleaved Caspase-1 expression.

3.7. APCP enhances the function of the central cholinergic system

As shown in Fig. 8A and B, the hippocampal AChE activity of model group was significantly increased, while the activity of the CHAT enzyme was decreased ($P < 0.05$). The acetylcholine metabolism-related enzymes (AChE and CHAT) changes were significantly reversed by APCP (30 mg/kg). The expression of AChE and CHAT in the hippocampus was also detected by western blot (Fig. 8C), which was in conformity with the results of Elisa (all $P < 0.05$).

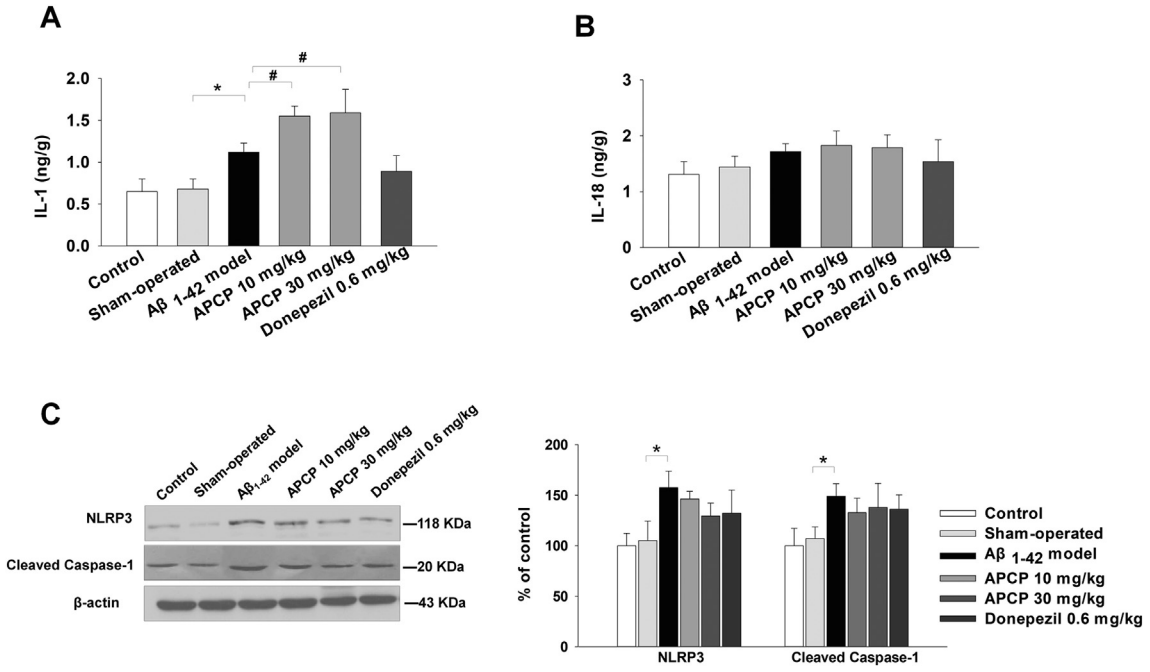

Fig. 7. Effect of APCP on the activation of NLRP3 inflammasome in hippocampus of $A\beta_{1-42}$-induced memory deficits mice. (A) The levels of IL-1β and IL-18 in the hippocampus were measured by ELISA. (B) Representative and quantitative western blot assay results, in which the lanes represent the NLRP3 and cleaved Caspase-1 protein expression in hippocampus. All data are expressed as means ± SEM ($n = 5–6$/group for Elisa experiment, $n = 3$/group for western blot). For statistical significance, $^{*}P < 0.05$ compared with the sham-operated (S-O) group; $^{\#}P < 0.05$ compared with $A\beta_{1-42}$ model group.

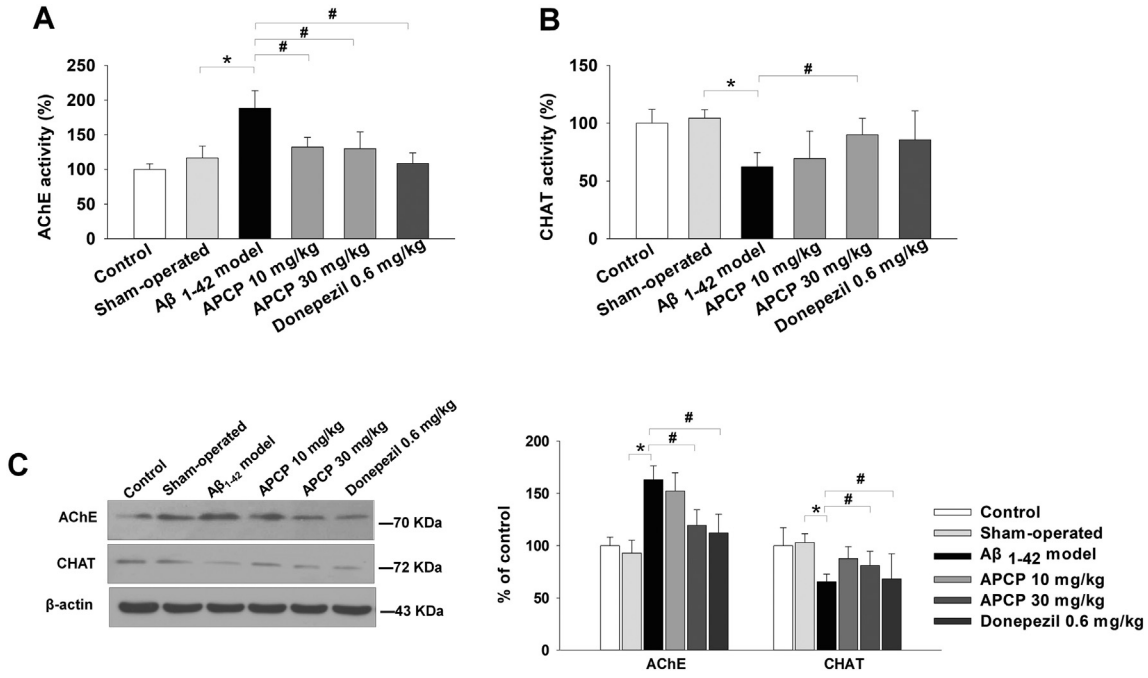

Fig. 8. Effect of APCP on the activity and protein expression of AChE and CHAT. (A) The activity of AChE and CHAT in the hippocampus were measured by ELISA. (B) Representative and quantitative western blot assay results, in which the lanes represent the AChE and CHAT protein expression in hippocampus. All data are expressed as means ± SEM ($n = 5$–6/group for Elisa experiment, $n = 3$/group for western blot). For statistical significance, $^{*}P < 0.05$ compared with the sham-operated (S-O) group; $^{\#}P < 0.05$ compared with $A\beta_{1-42}$ model group.

4. Discussion

The major finding of the present study was that, CD73 inhibitor APCP ameliorates learning and memory deficits by decreasing CD73-derived adenosine production and reinforce cholinergic functions in the hippocampus. The data showed that administration of APCP (30 mg/kg) enhanced the cognitive performances in behavior tests, including Y-maze Test and Morris water maze. Meanwhile, the microdialysis results showed that administration of APCP reversed Aβ-induced increase of extracellular adenosine content in the dialysate of hippocampus. In addition, APCP (10 and 30 mg/kg) showed no significant effect on NLRP3 inflammasome activation in hippocampus. Further, APCP could upregulate CHAT protein expression, while AChE downregulated, indicating that APCP enhances the function of the central cholinergic function in CNS.

Adenosine, a purine nucleoside, has drawn a lot of interest for its neuromodulatory activity in neurological diseases. Available evidences consistently show that inhibition of adenosine receptors has a cognitive protection effect in AD. For example, the A1-selective antagonist DPCPX could ameliorate memory disruption mediated by adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA) in the mouse passive avoidance test [17]. In addition, adenosine receptor antagonists 8-phenyltheophylline (8-PT) and theophylline inhibited the restoration of memory caused by adenosine receptor agonists cyclohexyladenosine (CHA) or N(6)-phenylisopropyladenosine (R-PIA) [18]. Moreover, the non-selective A-R antagonist caffeine and selective A2a receptor antagonist ZM-241385 could alleviate Aβ-induced neuronal apoptosis [10]. In the current study, we found that acute administration of $A\beta_{1-42}$ resulted in increased adenosine release in the hippocampus. Together with previous observation, these findings indicate that the purinergic system might be involved in AD and constitutes a new therapeutic target for AD. Consistent with previous studies, we suggested that inhibition of hippocampal adenosine production by CD73 inhibitor or adenosine receptor antagonists may have potential therapeutic effect in AD.

In contrast to the role of adenosine receptors, ATP receptor antagonists could impair learning and memory functions, while their agonists protect AD. Several studies have found that $P2Y_2$ receptor expression was reduced in autopsy of AD patients [19,20]. $P2Y_2$ receptor genes silencing increased mortality in AD mice, increased neurological deficits, and caused more accumulation of Aβ [21]. Moreover, Kim et al. found that activation of the $P2Y_2$ receptor enhanced phagocytosis and uptake of $A\beta_{1-42}$ by microglia, thereby reducing the formation of Aβ [22]. As extracellular adenosine is mainly derived from the metabolism of extracellular ATP and AMP through CD73, we suppose that CD73 may be the "equilibrium point" of the extracellular purinergic system, participates in the regulation of purinergic neurotransmitter release in the CNS and, therefore, it is expected to become a potential target for AD treatment. Intervention of CD73 may affect both P2Y receptors and adenosine receptors, that may have more potent therapeutic effect on AD. One of the limitations in the current study was the absence of ATP detection due to limitations of the instrument.

At present, it is well accepted that central inflammation considered to contribute to the pathology of AD [23,24]. The NLRP3 inflammasome, a complex containing the apoptosis-associated speck-like protein (ASC), caspase-1 and NLRP3, could be activated by Aβ in the central nervous system, leading to secretion of IL-1β and IL-18 [25]. Consistent with previous studies, the acquired data here suggests that Aβ stimulated the activation of NLRP3 inflammasome in the hippocampus of mice. Our findings also indicate that CD73 inhibitor APCP triggered the release of IL-1 in hippocampus. In agreement with this observation, Bynoe et al. [26] reported that colonic epithelia in CD73(-/-) mice exhibited high levels of IL-1β and constitutive activation of NF-κB. Another study on genetically deficient CD73 (-/-) mice demonstrated severe loss of blood brain barrier function with increased expression of IL-6, TNF-α and IL-1β [27].

Of note, our results indicated that APCP promoted the IL-1 release without affecting NLRP3 and caspase-1 protein expression. It is well documented that the extracellular ATP acting via the P2X7 receptor induces NLRP3 inflammasome-dependent release of IL-1β. Given the undisputed role of the NLRP3 inflammasome in the maturation and release of IL-1β, we speculate that secretion of IL-1 by APCP may not through P2XR, but more likely through attenuation the activation of

adenosine receptors through decreasing immunosuppressive adenosine production [28,29]. Studies over the last two decades have identified extracellular adenosine as a critical element in immune regulation. These studies noted a concomitant decrease in the release of pro-inflammatory cytokines, including IL-1β [30,31,32,33], INF-γ, TNF-α, IL-6, from its binding to A2A or A3 receptors [34]. Romio et al. [35] reported that activation inhibited the release of IL-1 by 39% in Teff cells. It has become clear that endogenous adenosine inhibits transmitter and cytokines release by suppressing Ca^{2+} influx through voltage-gated Ca^{2+} channels [36]. Accordingly, APCP promoted the secretion of IL-1 probably by reducing the production of immunosuppressive adenosine.

It is well known that cholinergic system in the brain plays an important role in cognitive function [37]. The degree of cognitive impairment in AD patients is closely related to the content of acetylcholine in the brain. Ach is catalyzed by acetyltransferase (ChAT) in cholinergic neurons and is rapidly hydrolyzed by acetylcholinesterase (AChE) after being transported through the axon to the synaptic cleft. Donepezil, an AChE inhibitor, could specifically inhibit the activity of AChE in the brain and inhibit the degradation of acetylcholine in the brain to improve the clinical symptoms of patients with dementia. The results of this study showed that the ChAT activity in the hippocampus of the model group mice was significantly reduced, while the AChE activity significantly increased. The administration of APCP resulted in an increase in ChAT activity and a significant decrease in AChE activity. This study shows that APCP can improve the learning and memory function of AD mice by increasing hippocampal ChAT activity and reducing AChE activity in the hippocampal cholinergic nervous system of dementia mice. There is evidence that ATP is released in the presynaptic membrane with the release of acetylcholine, while the metabolite adenosine from ATP inhibits the release of ATP and acetylcholine by acting on CD73 as a feedback inhibitor [38]. Therefore, we speculate that APCP may enhance cholinergic function by attenuating the negative feedback effect of adenosine.

5. Conclusion

In summary, we present here for the first time that inhibition of CD73 by APCP is of therapeutic value for AD, which may be due to the decrease of CD73-driven adenosine concentration in hippocampus. Improvement of cholinergic function of the central nervous system may also be involved in the effects of APCP.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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