



# Caffeine Functions by Inhibiting Dorsal and Ventral Hippocampal Adenosine 2A Receptors to Modulate Memory and Anxiety, Respectively

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As a nonspecific antagonist of the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R), caffeine enhances learning and improves memory impairment. Simultaneously, the consumption of caffeine correlates with a feeling of anxiety. The hippocampus is functionally differentiated along its dorsal/ventral axis and plays a crucial role both in memory and anxiety. Whether caffeine exerts its regulation by inhibiting A<sub>2A</sub>Rs in different subregions of the hippocampus is still unknown. In the present study, we found that after chronic intake of drinking water containing caffeine (1 g/L, 3 weeks), mice exhibited aggravated anxiety-like behavior and enhanced memory function. Tissue-specific, functional disruption of dorsal hippocampal A<sub>2A</sub>Rs by the CRE-LoxP system prevented the memory-enhancing effect of caffeine, while selective disruption of ventral hippocampal A<sub>2A</sub>Rs blocked the impact of caffeine on anxiety. These results, together with the enhanced memory of dorsal hippocampus A<sub>2A</sub>R knockout mice and greater anxiety-like behavior of ventral hippocampus A<sub>2A</sub>R knockout mice without caffeine, indicates a dissociation between the roles of ventral and dorsal hippocampal A<sub>2A</sub> receptors in caffeine's effects on anxiety-like and memory-related behavioral measures, respectively. Furthermore, optogenetic activation of dorsal or ventral hippocampal A<sub>2A</sub>Rs reversed the behavioral alterations caused by drinking caffeine, leading to impaired memory or decreased anxiety-like behaviors, respectively. Taken together, our findings suggest that the memory- and anxiety-enhancing effects of caffeine are related to the differential effects of inhibiting A<sub>2A</sub>Rs in the dorsal and ventral hippocampus, respectively.

**Keywords:** caffeine, dorsal hippocampus, ventral hippocampus, adenosine A<sub>2A</sub> receptor, memory, anxiety

## INTRODUCTION

As the most widely consumed psychotropic substance and a component of the most popular beverages, caffeine is used to counteract performance impairments associated with sleep loss (Irwin et al., 2020). The consumption of caffeine attenuates memory impairments associated with aging and Alzheimer's disease (AD) (Solfrizzi et al., 2015; Jacobson et al., 2020; Londzin, et al., 2021) and enhances memory in healthy humans (Borota et al., 2014; Irwin et al., 2020). However, controlled studies have confirmed that caffeine produces negative effects such as increased anxiety (Garcia and Salloum, 2015; Khurana and Bansal, 2019). Similarly, mouse studies have also shown that caffeine ingestion improves memory and induces anxiety (Xu and Reichelt, 2018). The main molecular

targets of caffeine in the brain are adenosine receptors, including the inhibitory A<sub>1</sub> receptor (A<sub>1</sub>R) and facilitatory A<sub>2A</sub> receptor (A<sub>2A</sub>R) (Ikram et al., 2020). From results obtained in animal models, it was further concluded that the effect of caffeine on both anxiety and memory performance was mimicked by the selective blockade of A<sub>2A</sub>, but not of A<sub>1</sub> receptor (Kaster et al., 2015; Machado et al., 2017).

The hippocampus belongs to the limbic system and plays an important role in memory, spatial navigation and emotion. The hippocampus can be divided into two segments along its longitudinal axis: the dorsal and ventral subregions. Early anatomical studies demonstrated differences between the afferent and efferent nerve projections of the dorsal hippocampus (dHPC) and ventral hippocampus (vHPC) (Swanson and Cowan, 1977). According to a current consensus, the role played by the most dorsally located hippocampal segment is on cognitive operations like spatial navigation, while internally monitoring functions related to emotionality are taken on by the ventral segment of the hippocampus (Trompoukis and Papatheodoropoulos, 2020). Moreover, some studies have found that caffeine reverts memory impairment in a depression-prone mouse strain with upregulation of adenosine A<sub>2A</sub> receptors in the hippocampus (Machado et al., 2017), while selective A<sub>2A</sub>R knockout in the forebrain region (striatum, hippocampus, and cortex) induces anxiety-like behavior (Wei et al., 2014). All the evidence suggests that the hippocampal adenosine A<sub>2A</sub> receptor is likely to emerge as an important receptor in the regulation of caffeine on memory and anxiety. However, whether the different effects of caffeine on overall function are derived from its action on adenosine A<sub>2A</sub> receptors in different subregions of the hippocampus is still unclear.

To explore the above question, we first verified that overall A<sub>2A</sub>R knockout reduced the effect of caffeine on mice. Furthermore, we found that localized knockout of dHPC A<sub>2A</sub>Rs reduced the regulation of caffeine on memory but did not affect anxiety, whereas localized knockout of vHPC A<sub>2A</sub>Rs reduced the regulation of caffeine on anxiety without affecting memory. To confirm the memory-enhancing effects of dHPC A<sub>2A</sub>R knockout, we assessed the level of SNAP-25, a synaptic marker reflecting synapse formation and remodeling (Batista et al., 2017). To confirm the anxiogenic effects of vHPC A<sub>2A</sub>R knockout, we assessed the level of SNAP-25, a glutamatergic-selective marker and labels excitatory glutamatergic neurons (Zheng et al., 2015; Martineau et al., 2017). Finally, specific stimulation of dorsal/ventral hippocampal A<sub>2A</sub>Rs by optogenetic techniques demonstrated that dorsal hippocampal A<sub>2A</sub>R activation impaired memory while ventral hippocampal A<sub>2A</sub>R activation reduced anxiety. Therefore, our experiments showed that caffeine regulates memory and anxiety by inhibiting the dorsal and ventral hippocampal A<sub>2A</sub>Rs, respectively.

## MATERIALS AND METHODS

### Animals

Adult male C57BL/6 mice (weighing 25–30 g, 11–13 weeks old) were purchased and used in our study. Global A<sub>2A</sub>R knockout

mice were established on a C57BL/6 background as our previously described (Zeng et al., 2020), and their littermates were used as wild-type (WT) mice in this experiment. Mice with a ‘floxed’ adenosine A<sub>2A</sub>R gene (A<sub>2A</sub><sup>fllox/fllox</sup> mice) created by insertion of loxP sequences into introns flanking an exon of the A<sub>2A</sub>R gene (Bastia et al., 2005; Yu et al., 2009) were provided by Dr. Chen. The experimental procedures were performed in accordance with the guidelines of the Animal Ethical and Welfare Committee of the Army Medical University.

### Viral Production

For conditional knockout (Cre/loxP system) of neuronal A<sub>2A</sub>R, pAAV-hSyn-EGFP-2A-CRE virus and pAAV-hSyn-MCS-EGFP-3Flag virus (control virus) were packaged and supplied by OBiO Technology (Shanghai) Corp. Ltd.

For optogenetic manipulations of neuronal A<sub>2A</sub>R, a chimeric rhodopsin-A<sub>2A</sub>R protein (optoA<sub>2A</sub>R) was developed by replacing the intracellular domain of rhodopsin with that of A<sub>2A</sub>R as described in our previous research (Li et al., 2015). Extracellular adenosine or caffeine cannot react with optoA<sub>2A</sub>R. Similarly, pAAV-CaMKIIa-optoA<sub>2A</sub>R-mCherry virus and pAAV-CaMKIIa-MCS-mCherry-3FLAG virus (control virus) were constructed and supplied by OBiO Technology (Shanghai) Corp. Ltd. All viruses were used at titers of ~4–8\*10<sup>12</sup> vg/ml.

### Drug Treatments

In the first part of our experiment, mice (knockout or wild-type) were randomly allocated to two groups: a control group provided with drinking water without drug and a treatment group provided with drinking water containing caffeine (1 g/L, Sigma) starting 3 weeks before behavioral tests. This dose and schedule of administration of caffeine was chosen since it was sufficient to improve memory and increase anxiety according to a previous study (Kaster et al., 2015).

In the second part of the study, A<sub>2A</sub><sup>fllox/fllox</sup> mice were injected with CRE virus or EGFP virus. Half the mice in each group were then provided with caffeine-containing drinking water starting 3 weeks before behavioral tests, and the other half were provided with normal drinking water.

In the last part, wild-type mice were randomized into two groups: an experimental group injected with the optoA<sub>2A</sub>R virus and a control group were injected with the mCherry virus. Then, all mice underwent optical fiber implantation and were treated with caffeine starting 3 weeks before optogenetic manipulation.

In all experiments, the drinking water with or without caffeine were provided every day, until the mice were killed.

### Animal Surgery: Virus Injection and Optical Fiber Implantation

For all surgical procedures, mice were anesthetized with 1.5% isoflurane at an oxygen flow rate of 1 L/min and then immobilized in a Robot Stereotaxic apparatus (Neurostar, Tübingen, Germany). The fur was shaved, and a midline scalp incision was made for surgical procedures. After surgery, the mice

were given saline containing buprenorphine (0.13 mg/kg, subcutaneously) for 3 days for analgesia.

For brain region-specific knockout of  $A_{2A}R$ , 1.5  $\mu$ L pAAV-hSyn-EGFP-2A-CRE virus (or control virus) was injected into the each dHPC (AP: 1.1 mm; ML:  $\pm$ 1.25 mm; DV: +1.75 mm) or each vHPC (AP: 3.4 mm; ML:  $\pm$ 2.5 mm; DV: +4.0 mm) of  $A_{2A}^{flox/flox}$  mice (Total 3.0  $\mu$ L).

To express opto $A_{2A}R$  in hippocampal neurons, we injected 1.5  $\mu$ L pAAV-CaMKIIa-Opto $A_{2A}$  (A400S)-mCherry virus (or control virus) into the left dorsal or ventral subregion (viral injection coordinates are described above). Four weeks after injection, a 200  $\mu$ m optical fiber (Shanghai Fiblaser Technology Co., Ltd.) was implanted in the same coordinates. Via a patch cable, the optical fiber was connected to a 473 nm DPSS laser (100 mW; Shanghai Laser and Optics Century). The power density at the fiber tip was approximately 5 mW/mm<sup>2</sup>, and light was delivered with a 50 m pulse width (10Hz). For the optogenetic experiment, mice were habituated with an optical fiber connected to the optical patch cable without laser stimulation for 30 min before the behavior tests, and optical stimulation was delivered specifically according to the different behavioral tests (see below). For immunofluorescence analyses, mice were euthanized by cervical dislocation following 10 min of optical stimulation.

## Behavioral Experiments

To eliminate the acute effects of caffeine while preserving its long-term chronic effects, all behavioral tests were performed within 24–48 h after 3-week caffeine treatment.

**Open Field Test:** Mice were placed in a square chamber (40 cm  $\times$  40 cm  $\times$  40 cm length-width-height) with a dimly lit (approximately 65 lux). The center zone was a 20  $\times$  20 cm square. Mice were allowed to freely explore the environment for 5 min, while activities were recorded and analyzed by EthoVision XT behavioral tracking software (Noldus Information Technology Inc.). For optogenetic manipulation, light stimulation was maintained for the entire 5-min duration of the open-field test.

**Elevated Plus Maze (EPM) Test:** The elevated plus maze had open and closed arms (30 cm  $\times$  8.5 cm length-width, 20 cm tall closed arms) that extended from a central platform (8.5  $\times$  8.5 cm). The maze was placed at a height of 58 cm and the room light was about 65 lux. The mouse was placed in the central platform of the maze facing an open arm and were allowed to freely explore the EPM for 5 min, and their activities were recorded and analyzed by EthoVision software (Noldus Information Technology Inc.). For optogenetic experiments, light stimulation was used for a total period of 5 min while mice explored the EPM.

**Novel Object Recognition (NOR) Test:** NOR test was carried out in a home-cage arena (45 cm  $\times$  45 cm  $\times$  50 cm length-width-height) which they were allowed to freely explore for 10 min on the previous day. In the training trial on the next day, mice were presented with two same objects (a red cube, 2.8 cm  $\times$  2.8 cm  $\times$  2.8 cm) placed in opposite corners for 10 min. The exploration of the objects, defined by mice showing investigative behaviors (head orientation or sniffing) or playing within 1 cm around the object, was measured. In the testing trial (24 h later), one of the

identical objects was changed for a novel object (a yellow pyramid, height 3.0 cm, base 2.8 cm  $\times$  2.8 cm), and the animals were left in the cage for 10 min. The exploration time for the familiar and the novel object during the test phase was recorded with EthoVision software. For the optogenetic experiment, light stimuli were only delivered during the testing trial.

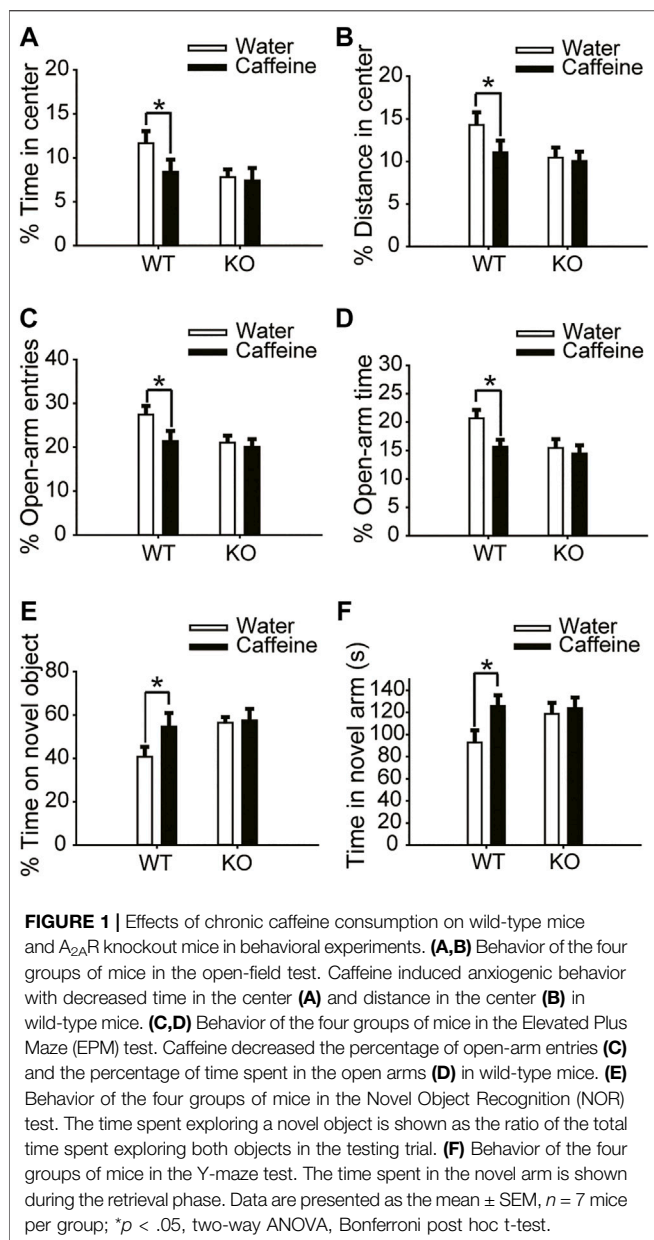
**Y-Maze Test:** Y-maze test was carried out in a gray maze formed by three arms (28 cm  $\times$  8.5 cm  $\times$  20 cm length-width-height, 58 cm above the ground) so as to form a Y shape. Each mouse was first allowed to explore the maze for 5 min while one arm was blocked (acquisition phase). After 2 h, mice had access to all three arms for a 5 min period (retrieval phase). During the second period, the time spent in each arm was measured by a video-tracking system (Noldus Information Technology Inc.). For the optogenetic experiment, light stimuli were only presented during the retrieval trial.

## Immunofluorescence

Following behavioral experiments or light stimulation, mice were sequentially perfused with saline and 4% paraformaldehyde in PBS. Brains were postfixed, and coronal sections (30  $\mu$ m) were cut and prepared for immunofluorescence. Free-floating sections were washed in PBS and then incubated for 30 min in 0.3% Triton X-100 and 3% BSA or goat serum. Sections were incubated with the following primary antibodies overnight at 4°C: anti- $A_{2A}R$  (1:200, Frontier Institute, AB\_2571655), anti-c-Fos (1:50, Santa Cruz, sc-271243), anti-synaptosomal-associated protein 25 (SNAP-25; 1:100, Abcam, ab5666) and anti-vesicular glutamate transporter 1 (vGluT1; 1:100, Abcam, ab227805). Sections were then washed with PBS and incubated with fluorescence-tagged secondary antibodies including Cy3 (1:500, donkey anti-goat, Abcam, ab6949), Cy3 (1:500, goat anti-mouse, Abcam, ab97035), Cy3 (1:500, goat anti-rabbit, Abcam, ab6939), and Alexa Fluor 488 (1:500, goat anti-mouse, Abcam, ab150117) for 1 h at 37°C. Nuclei were subsequently stained with DAPI (Santa Cruz, sc-359850). High-magnification images were captured using a confocal laser-scanning microscope (Leica TCS-SP2, laser lines at 488, 543, 633) and analyzed with Image-Pro Plus 4.5 software. For quantification of immunofluorescence, at least 50 cells were evaluated in each field (3 fields per slice, three slices per mouse, three mice from each analyzed group).

## Statistical Analysis

Results are expressed as the means  $\pm$  SEM. All semi-quantitative assessments of histological staining were made by a single investigator blinded to the genotype and treatment of the experimental animals. Sample size was chosen according to previous reports and our pre-experiments. Two-way analyses of variance (ANOVAs) were used to assess the effects of caffeine, gene manipulation and the caffeine  $\times$  gene manipulation interaction in the  $A_{2A}R$  total (or region-specific) knockout mice. Two-way ANOVAs were used to assess the effects of opto $A_{2A}R$  virus, light stimulation and the opto $A_{2A}R$  virus  $\times$  light stimulation interaction in the opto $A_{2A}R$  mice. A value of  $p < .05$  was considered statistically significant.



## RESULTS

### Global $A_{2A}R$ Knockout Blocked the Effect of Caffeine on Anxiety and Memory

The open-field test and EPM test were used to evaluate anxiety-like behaviors. Chronic caffeine consumption (3 weeks) significantly reduced the time (Figure 1A, for caffeine,  $F_{(1,28)} = 5.741, p < .01$ ; for knockout,  $F_{(1,28)} = 16.341, p < .01$ ; for caffeine  $\times$  knockout interaction,  $F_{(3,28)} = 4.124, p < .01$ ) and distance (Figure 1B, for caffeine,  $F_{(1,28)} = 56.114, p < .001$ ; for knockout,  $F_{(1,28)} = 23.554, p < .01$ ; for caffeine  $\times$  knockout interaction,  $F_{(3,28)} = 4.451, p < .01$ ) in the center of the open field, and had no significant effect on the total distance (Supplementary Figure S1A, for caffeine,  $F_{(1,28)} = 0.372, p = .547$ ; for knockout,  $F_{(1,28)} = .001, p = .975$ ; for caffeine  $\times$  knockout interaction,  $F_{(3,28)} = 0.985, p = 0.331$ ). In the EPM,

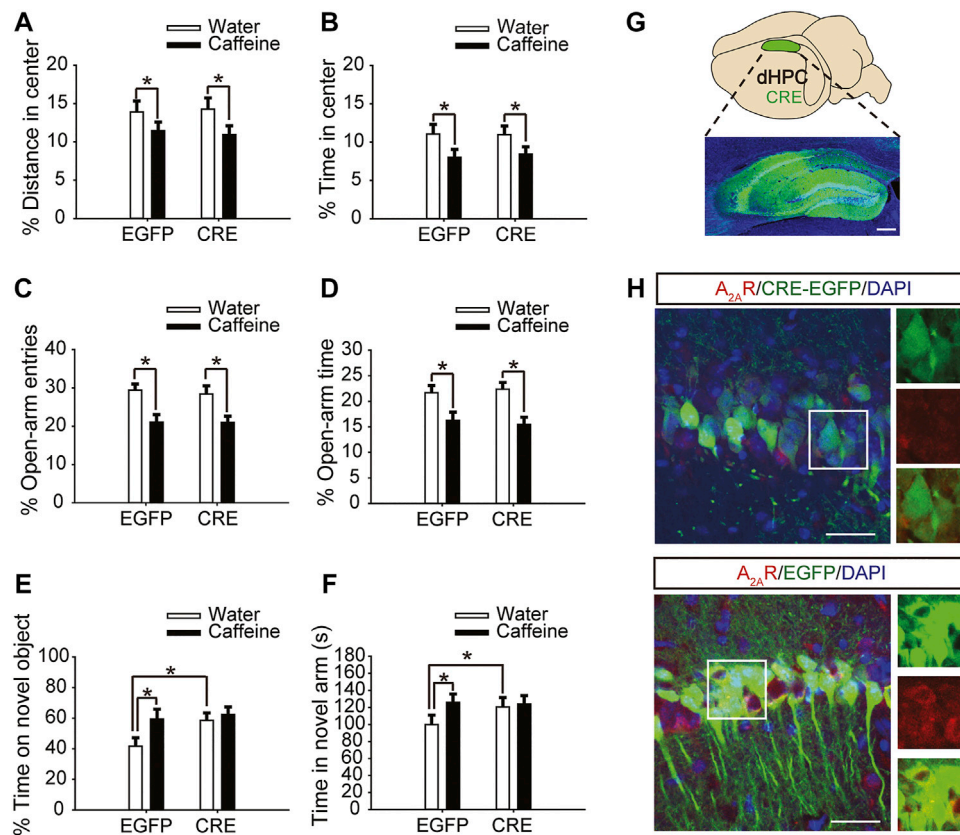
caffeine treatment decreased the percentage of time (Figure 1D, for caffeine,  $F_{(1,28)} = 9.125, p < .01$ ; for knockout,  $F_{(1,28)} = 21.934, p < .01$ ; for caffeine  $\times$  knockout interaction,  $F_{(3,28)} = 23.344, p < .01$ ) and number of entries (Figure 1C, for caffeine,  $F_{(1,28)} = 14.624, p < .01$ ; for knockout,  $F_{(1,28)} = 21.679, p < .01$ ; for caffeine  $\times$  knockout interaction  $F_{(3,28)} = 8.824, p < .01$ ) in the open arms which confirmed the open-field test results. We next examined object recognition to evaluate the role of caffeine in memory formation. The caffeine-treated mice showed greater exploration of the novel object over the familiar object (Figure 1E, for caffeine,  $F_{(1,28)} = 12.347, p < .01$ ; for knockout,  $F_{(1,28)} = 15.472, p < .01$ ; for caffeine  $\times$  knockout interaction,  $F_{(3,28)} = 14.147, p < .01$ ). The spatial recognition memory of mice was probed using a two-trial Y-maze paradigm. Likewise, caffeine-treated mice showed increased time in the novel arm compared with control mice (Figure 1F, for caffeine,  $F_{(1,28)} = 22.712, p < .01$ ; for knockout,  $F_{(1,28)} = 15.755, p < .01$ ; for caffeine  $\times$  knockout interaction,  $F_{(3,28)} = 21.457, p < .01$ ). This finding suggests that chronic caffeine consumption for 3 weeks enhanced memory performance in mice.

However, chronic caffeine consumption did not alter anxiety-like behaviors in global  $A_{2A}R$  knockout mice. There were no significant differences between caffeine-treated mice (caffeine + knockout) and control mice (water + knockout) in either the open-field test or the EPM test (Figure 1C and Figure 1D). At the same time,  $A_{2A}R$  knockout blocked the effect of caffeine in the NOR test and Y-maze test (Figures 1E,F), suggesting that the effect of caffeine on memory was prohibited by  $A_{2A}R$  inactivation. It is worth noting that in global  $A_{2A}R$  knockout mice, there were baseline differences in behaviors that are independent of caffeine (Figures 1A–F). But the costimulation with caffeine and  $A_{2A}R$  knockout did not produce additive effects which reflected the interaction between caffeine- $A_{2A}R$  function.

### Dorsal Hippocampal $A_{2A}R$ Knockout Blocked the Caffeine-Induced Enhancement of Memory

Six weeks after the injection of pAAV-syn-EGFP-2A-CRE virus in the dHPC of  $A_{2A}^{lox/lox}$  mice (Figure 2G), the  $A_{2A}R$  level was significantly reduced (Figure 2H). Then, after drinking caffeine for 3 weeks, mice showed reduced center distance (Figure 2A, for caffeine,  $F_{(1,28)} = 7.473, p < .01$ ; for CRE,  $F_{(1,28)} = 17.453, p = .413$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 32.913, p = .302$ ) and center time (Figure 2B, for caffeine,  $F_{(1,28)} = 15.417, p < .01$ ; for CRE,  $F_{(1,28)} = 32.348, p = .235$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 42.374, p = .328$ ) in the open-field test, and had no significant effect on the total distance (Supplementary Figure S1B, for caffeine,  $F_{(1,28)} = .042, p = 0.839$ ; for CRE,  $F_{(1,28)} = 1.817, p = .190$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 0.767, p = .389$ ). At the same time, caffeine reduced the number of entries (Figure 2C, for caffeine,  $F_{(1,28)} = 5.113, p < .01$ ; for CRE,  $F_{(1,28)} = 15.457, p = .191$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 21.479, p = .208$ ) and duration of time (Figure 2D, for caffeine,  $F_{(1,28)} = 3.323, p < .01$ ; for CRE,  $F_{(1,28)} = 16.278, p = .058$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 22.143, p = .572$ ) spent in the open arm of the EPM. These results indicate that





**FIGURE 2 |** Selective expression of pAAV-CRE in the dHPC triggers memory enhancement. **(A,B)** Behavior of the four groups of mice in the open-field test. Caffeine decreased the time in the center **(B)** and distance in center **(A)** in CRE and EGFP mice. **(C,D)** Behavior of the four groups of mice in the Elevated Plus Maze (EPM) test. Caffeine decreased the percentage of open-arm entries **(C)** and the percentage of time spent in the open arms **(D)** in CRE and EGFP mice. **(E)** Behavior of the four groups of mice in the Novel Object Recognition (NOR) test. The time spent exploring a novel object was increased by caffeine only in EGFP mice during the testing trial. **(F)** Behavior of the four groups of mice in the Y-maze test. The time spent in the novel arm was increased by caffeine only in EGFP mice during the retrieval phase. **(G)** Selected expression of AAV-CRE in the dHPC (scale bar = 500  $\mu$ m). **(H)** Images of hippocampal brain sections obtained from mice injected with AAV-CRE or AAV-EGFP (scale bar = 30  $\mu$ m). The level of A<sub>2A</sub>R (red) was lower in CRE-positive cells (upper panel) than in cells transfected with pAAV-EGFP (lower panel). Data are presented as the mean  $\pm$  SEM; n = 7 mice per group; \*p < .05, two-way ANOVA, Bonferroni post hoc t-test.

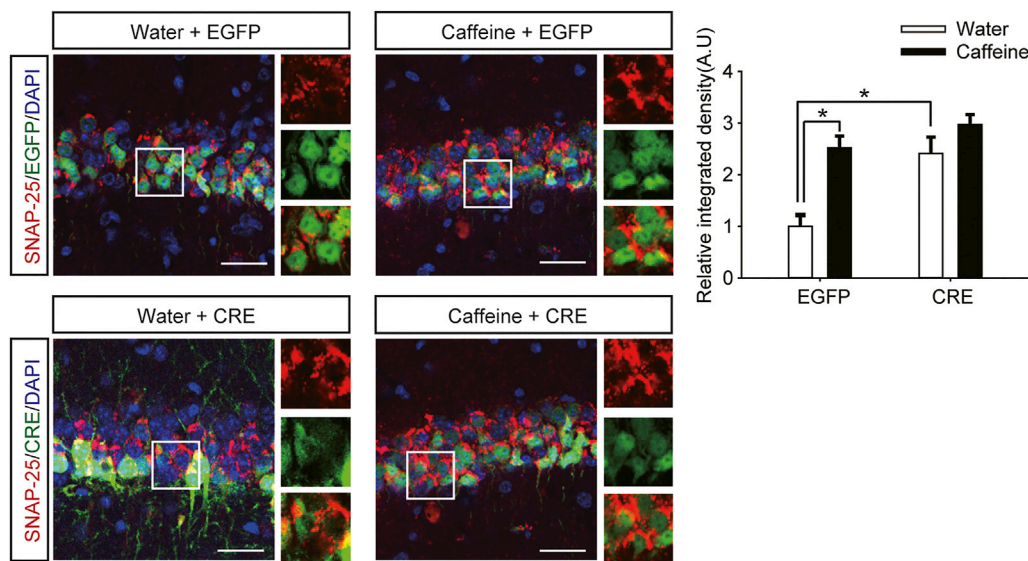
dHPC A<sub>2A</sub>R knockout did not affect the caffeine-induced anxiety-like behavior in mice.

However, after inactivation of dorsal hippocampal A<sub>2A</sub>Rs, there was no significant difference in behavior in the NOR test (**Figure 2E**, for caffeine,  $F_{(1,28)} = 1.413, p < .01$ ; for CRE,  $F_{(1,28)} = 19.413, p < .01$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 15.439, p < .01$ ) or Y-maze test (**Figure 2F**, for caffeine,  $F_{(1,28)} = 17.435, p < .01$ ; for CRE,  $F_{(1,28)} = 10.235, p < 0.01$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 5.636, p < .01$ ) between the caffeine-treated group and the control group, indicating that dHPC A<sub>2A</sub>R knockout eliminated the effect of caffeine on memory. Immunofluorescence results showed that the level of SNAP-25 in the pAAV-EGFP group given caffeine (EGFP + caffeine) was significantly higher than that in the group given water (EGFP + water). However, caffeine did not alter the expression of SNAP-25 in the pAAV-CRE group (**Figure 3**). Interestingly, the NOR test and Y-maze test showed that dorsal hippocampal A<sub>2A</sub>R knockout alone improved memory similar to chronic caffeine consumption (**Figures 2E,F**), and these behavioral changes were in accordance

with the trend observed in SNAP-25 expression (**Figure 3**). This evidence suggests that the effect of caffeine on memory in mice is derived from its action on dorsal hippocampal A<sub>2A</sub>Rs.

### Ventral Hippocampal A<sub>2A</sub>R Knockout Prevented Caffeine-Induced Anxiety

After expression of pAAV-syn-EGFP-2A-CRE in the vHPC (**Figure 4G**), chronic caffeine consumption for 3 weeks had no significant effect on behavior in the open-field test or EPM test (**Figures 4A–D**), indicating that inactivation of vHPC A<sub>2A</sub>Rs abolished the anxiogenic effect of caffeine. At the same time, mice in the pAAV-CRE group (CRE + water) showed greater anxiety-like behavior than pAAV-EGFP mice (EGFP + water) in the open-field test (**Figure 4A**, for caffeine,  $F_{(1,28)} = 15.445, p < .01$ ; for CRE,  $F_{(1,28)} = 17.609, p < .01$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 5.235, p < .01$ ; **Figure 4B**, for caffeine,  $F_{(1,28)} = 7.143, p < .01$ ; for CRE,  $F_{(1,28)} = 2.439, p < .01$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 8.833, p < .01$ ) and EPM test (**Figure 4C**,



**FIGURE 3 |** Selective deletion of dHPC  $A_{2A}R$ s improves SNAP-25 expression. Caffeine promoted SNAP-25 expression more in mice with the dHPC transfected with pAAV-EGFP than in mice transfected with pAAV-CRE. pAAV-CRE transfection also produced an additive effect on SNAP-25 levels independently. Representative images (left) and data (right) are presented as the mean  $\pm$  SEM;  $n = 27$  fields per group (3 fields per section, three sections per mouse, three mice per group). Scale bar = 30  $\mu$ m.

for caffeine,  $F_{(1,28)} = 11.415$ ,  $p < .01$ ; for CRE,  $F_{(1,28)} = 11.961$ ,  $p < .01$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 9.675$ ,  $p < .01$ ; **Figure 4D**, for caffeine,  $F_{(1,28)} = 21.435$ ,  $p < .01$ ; for CRE,  $F_{(1,28)} = 19.495$ ,  $p < .01$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 5.195$ ,  $p < .01$ , and had no significant effect on the total distance in the open-field test (**SupplementaryFigure S1C**, for caffeine,  $F_{(1,28)} = 0.108$ ,  $p = .746$ ; for CRE,  $F_{(1,28)} = 1.838$ ,  $p = .188$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 1.367$ ,  $p = .252$ ). The levels of vGluT1 in the four groups of mice (**Figure 5**) were consistent with the trend in behavior. Thus, these findings indicate that the anxiogenic effect of chronic caffeine consumption stems from its inhibition of ventral hippocampal  $A_{2A}R$ s.

Caffeine in drinking water still increased the time spent exploring a novel object (**Figure 4E**, for caffeine,  $F_{(1,28)} = 3.239$ ,  $p < .01$ ; for CRE,  $F_{(1,28)} = 13.319$ ,  $p = .279$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 21.173$ ,  $p = .064$ ) and the exploration time of the new arm (**Figure 4F**, for caffeine,  $F_{(1,28)} = 7.631$ ,  $p < .01$ ; for CRE,  $F_{(1,28)} = 23.659$ ,  $p = .288$ ; for caffeine  $\times$  CRE interaction,  $F_{(3,28)} = 31.849$ ,  $p = 0.233$ ) in both the pAAV-CRE and pAAV-EGFP mice, indicating that the ablation of ventral hippocampal  $A_{2A}R$ s did not affect the memory-enhancing effect of caffeine.

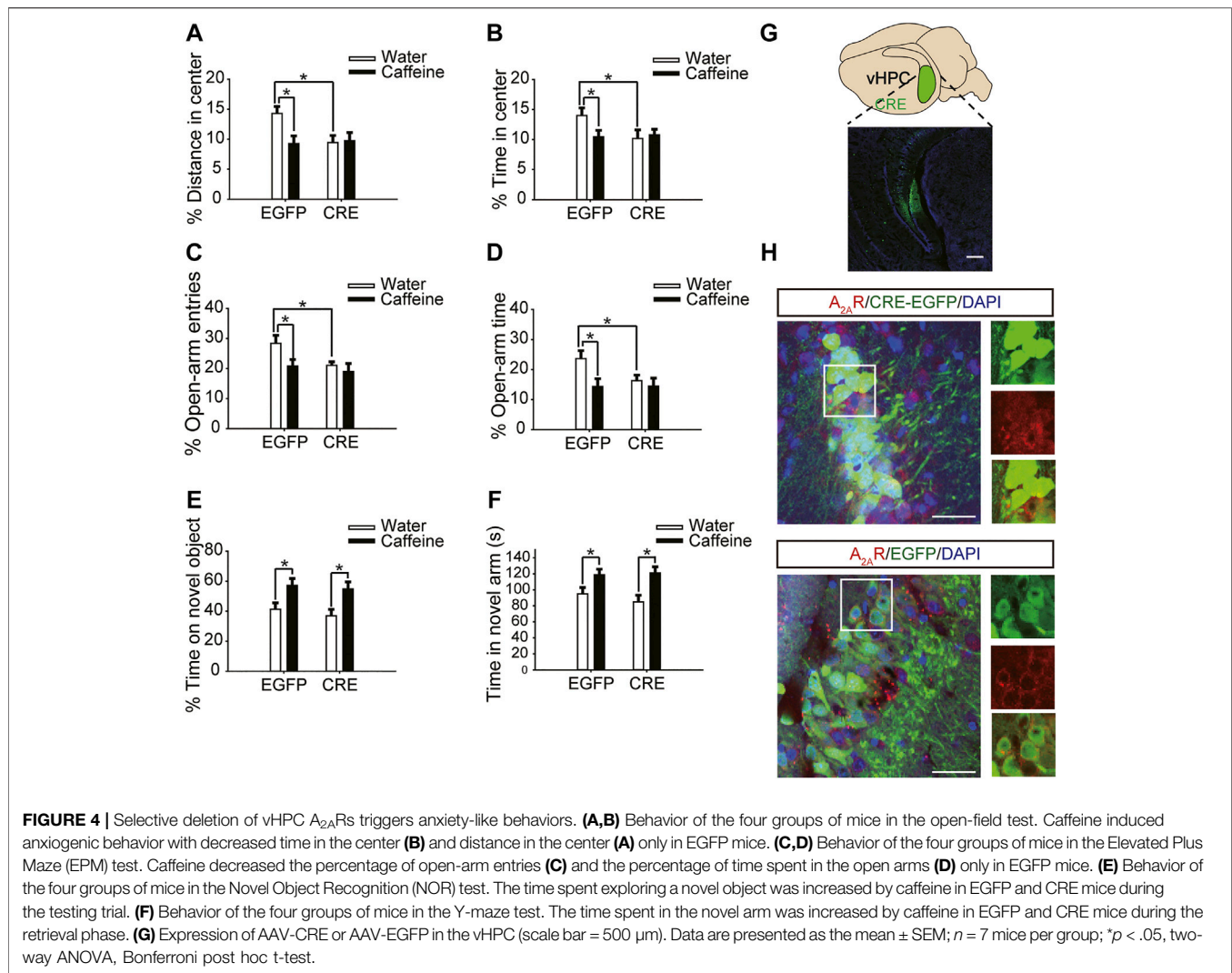
### Brain Region-specific Activation of dHPC/vHPC $A_{2A}R$ s Reversed the Regulation of Caffeine on Memory and Anxiety

Six weeks after dHPC/vHPC injection of pAAV-CaMKIIa-opto $A_{2A}R$ -mCherry (**Figures 6A,7A**), mice were exposed to caffeine in drinking water for 3 weeks. Light stimulation for 5 min significantly increased the levels of c-Fos in pAAV-opto $A_{2A}R$  mice but not in pAAV-mCherry mice (**Figure 6D**),

indicating that illumination was sufficient to activate the opto $A_{2A}R$  signaling pathway.

After optogenetic opto $A_{2A}R$  activation in the dHPC, both the time spent exploring novel objects (**Figure 6B**, for opto $A_{2A}R$  virus,  $F_{(1,28)} = 3.891$ ,  $p = .105$ ; for light stimulation,  $F_{(1,28)} = 11.391$ ,  $p < .05$ ; for virus  $\times$  light interaction,  $F_{(3,28)} = 25.317$ ,  $p = .091$ ) and time spent in the novel arm (**Figure 6C** for opto $A_{2A}R$  virus,  $F_{(1,28)} = 16.132$ ,  $p = .067$ ; for light stimulation,  $F_{(1,28)} = 13.639$ ,  $p < .05$ ; for virus  $\times$  light interaction,  $F_{(3,28)} = 12.558$ ,  $p = .334$ ) were significantly decreased, indicating that specific activation of dorsal hippocampal  $A_{2A}R$ s triggered obvious memory impairments in mice exposed to caffeine and further suggesting that caffeine promotes memory function by inhibiting dorsal hippocampal  $A_{2A}R$ s.

In addition, light activation of opto $A_{2A}R$  in the vHPC significantly enhanced the proportion of time spent in the center and distance in the center in the open-field test (**Figure 7B**, for opto $A_{2A}R$  virus,  $F_{(1,28)} = 3.229$ ,  $p = .255$ ; for light stimulation,  $F_{(1,28)} = 8.296$ ,  $p < .01$ ; for virus  $\times$  light interaction,  $F_{(3,28)} = 26.539$ ,  $p = .539$ ; **Figure 7C**, for opto $A_{2A}R$  virus,  $F_{(1,28)} = 11.453$ ,  $p = .548$ ; for light stimulation,  $F_{(1,28)} = 13.231$ ,  $p < .05$ ; for virus  $\times$  light interaction,  $F_{(1,28)} = 23.665$ ,  $p = .792$ ), and had no significant effect on the total distance (**Figure S1D** for opto $A_{2A}R$  virus,  $F_{(1,28)} = 0.299$ ,  $p = .589$ ; for light interaction,  $F_{(1,28)} = 0.088$ ,  $p = .769$ ; for virus  $\times$  light interaction,  $F_{(3,28)} = 0.246$ ,  $p = .624$ ). Furthermore, the number of open-arm entries and duration of time spent in the open arms in the EPM increased markedly with light activation (**Figure 7D**, for opto $A_{2A}R$  virus,  $F_{(1,28)} = 21.238$ ,  $p = .459$ ; for light stimulation,  $F_{(1,28)} = 17.487$ ,  $p < .001$ ; for virus  $\times$  light interaction,  $F_{(3,28)} = 28.292$ ,  $p = .428$ ; **Figure 7E**, for opto $A_{2A}R$  virus,  $F_{(1,28)} = 3.241$ ,  $p = .536$ ; for light stimulation,  $F_{(1,28)} = 14.549$ ,  $p < .01$ ; for virus  $\times$  light



interaction,  $F_{(3,28)} = 13.449$ ,  $p = .413$ ). These findings indicate that specific activation of ventral hippocampal  $A_{2A}$ Rs inhibited the anxiety-like behaviors of mice treated with caffeine and affirm that caffeine-induced anxiety originates from its suppression of ventral hippocampal  $A_{2A}$ Rs.

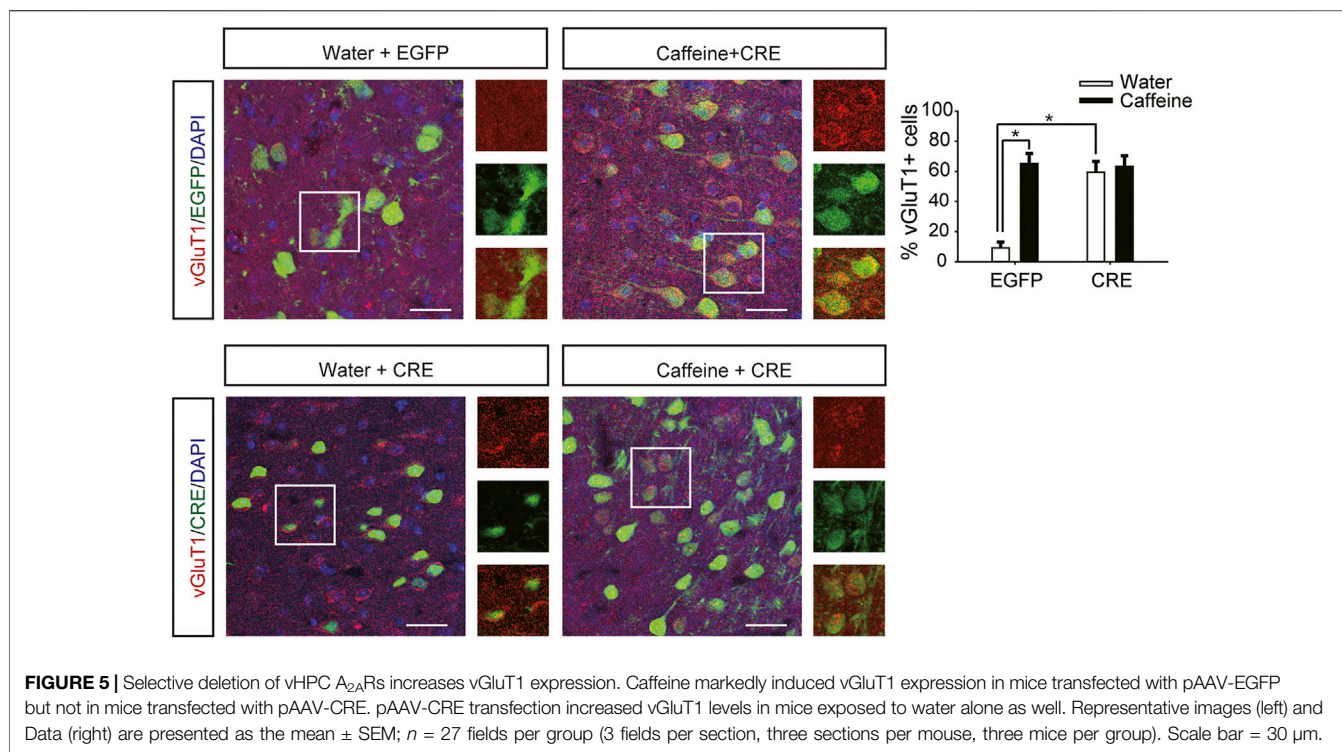
## DISCUSSION

In the present study, we demonstrated that chronic caffeine consumption (1 g/L, 3 weeks) increased anxiety-like behavior, as assessed by the open-field test and EPM, and enhanced memory, as reflected in the NOR and Y-maze. Consistent with our results, caffeine has been shown to reverse cognitive impairments in aging and Alzheimer's disease (AD) (Solfrizzi et al., 2015; Londzin et al., 2021) and lead to depression and anxiety-like behaviors (Richards and Smith, 2015; Mikkelsen et al., 2017). Acute caffeine consumption is widely used to counteract mood and performance impairments associated with sleep loss (Irwin et al., 2020). To eliminate the acute

effects of caffeine while preserving its long-term chronic effects, all behavioral tests were performed after 3-week caffeine treatment. Chronic but not acute treatment with caffeine is considered to induce those behavioral alterations. As caffeine is most widely consumed all over the world, our exploration of chronic caffeine consumption is more meaningful. Moreover, caffeine seemed to have no effects on anxiety and memory in our total  $A_{2A}$ R knockout mice. Statistical analysis also showed the strong interactions between caffeine and  $A_{2A}$ R function, which suggests that caffeine functions through the antagonism of  $A_{2A}$ Rs. The main targets for caffeine in the brain are the inhibitory  $A_1$ R and the facilitatory  $A_{2A}$ R (Fredholm et al., 2005). Which is different from anti-inflammatory effect of  $A_1$ R against noxious brain conditions (Martins et al., 2015),  $A_{2A}$ R may play a more important role in memory and anxiety.

Many evidence suggests that pathological brain conditions associated with memory impairment are accompanied by a local increase of the extracellular levels of adenosine (Chen et al., 2013) and an up-regulation and aberrant signaling of the brain  $A_{2A}$ R





(Cunha and Agostinho, 2010; Chen et al., 2013). Thus, A<sub>2A</sub>R blockade could attenuate the impairment of brain function (Cunha, 2016) and memory function in particular (Cunha and Agostinho, 2010). However, whether A<sub>2A</sub>R blockade increases learn and memory in healthy animals remains contentious. Several groups reported that A<sub>2A</sub>R blockade and caffeine did not increase memory performance in control rodents, whereas stressed mice displayed increased memory performance upon caffeine consumption or upon blocking A<sub>2A</sub>R (Prediger et al., 2005; Kaster et al., 2015; Laurent et al., 2016; Carvalho et al., 2019). But genetic KO studies have shown that inactivation of A<sub>2A</sub>R is sufficient to improve memory in healthy animals (Zhou et al., 2009; Wei et al., 2011). The mechanism by which genetic inactivation of A<sub>2A</sub>R strengthens memory is not clear. Loss of A<sub>2A</sub>R may impact cortical function through neuronal networks such as basal ganglia loop (Zhou et al., 2009) or potentiate striatal dopaminergic signaling via D<sub>2</sub>R to produce the memory enhancement (Wei et al., 2011).

While studies have shown that intraperitoneal injection of the A<sub>2A</sub>R agonist CGS21680 produces strong anxiety-like behavior (El Yacoubi et al., 2000), in our study, the A<sub>2A</sub>R knockout mice showed greater anxiety and enhanced memory compared to wild-type littermates. Considering that A<sub>2A</sub>R is widely distributed in the brain, the overall effect of drinking caffeine is likely to be a superposition of its antagonism towards A<sub>2A</sub>R in multiple brain regions. Therefore, A<sub>2A</sub>R in a particular brain subregion may be responsible for caffeine's effects on anxiety-like and memory-related behaviors.

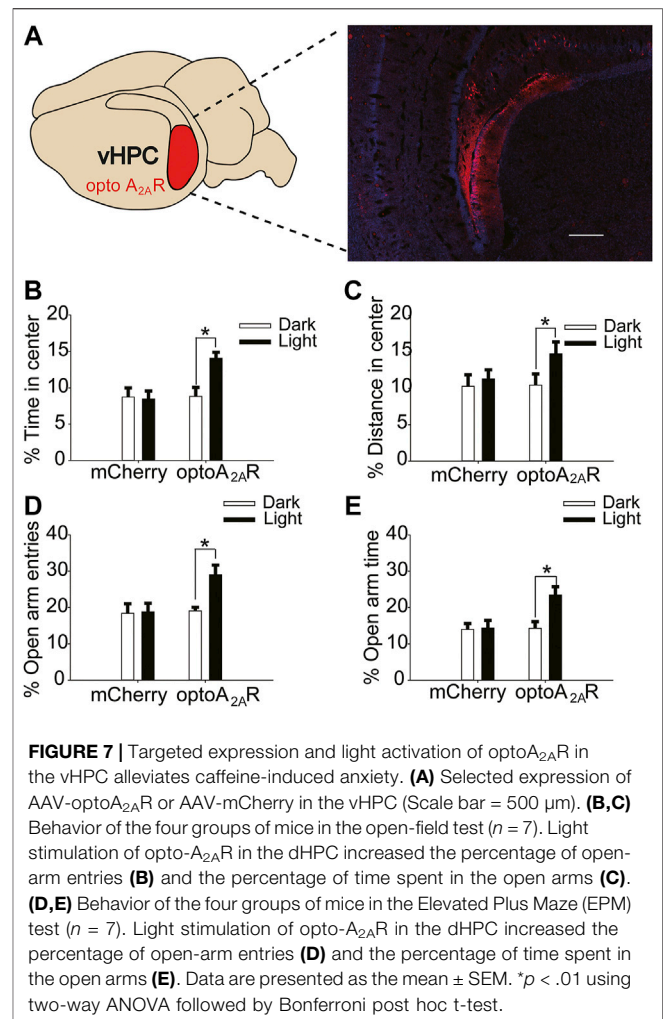
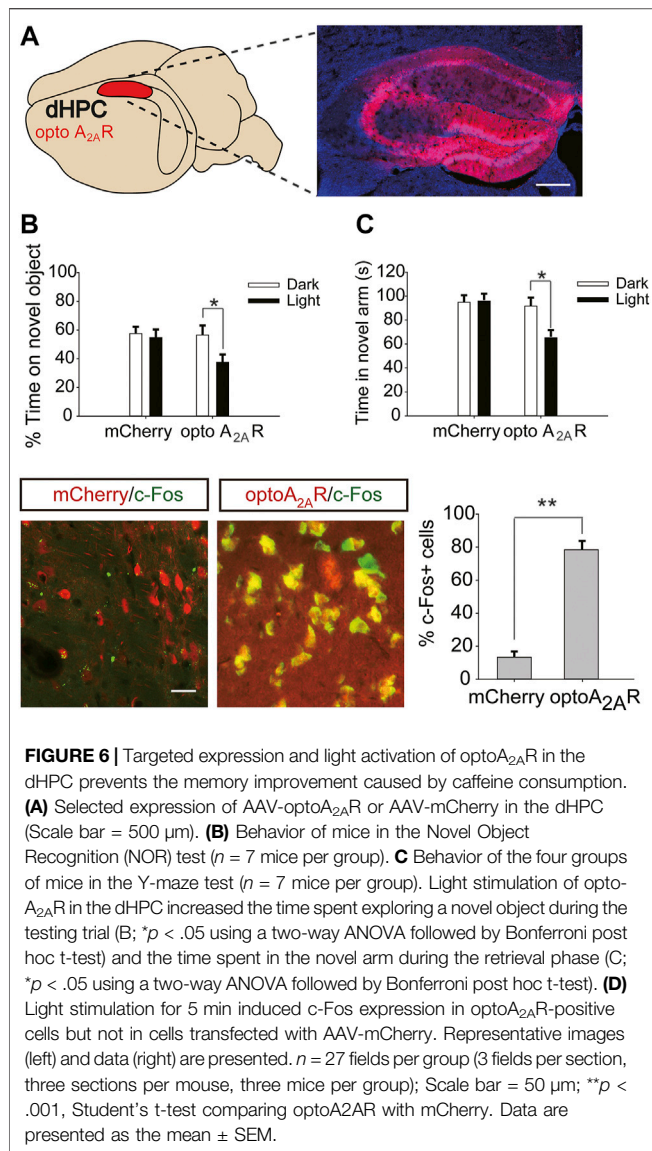
Another major advance provided by this study is the dissociation of the roles of the ventral and dorsal hippocampal A<sub>2A</sub>R receptors in caffeine's effects on anxiety-like and memory-

related behavioral measures, respectively, which are also consistent with the reported roles of the dorsal and ventral hippocampus more generally (Fanselow and Dong, 2010; McHugh et al., 2011; Schumacher et al., 2018). We discovered that knocking out dorsal hippocampal A<sub>2A</sub>Rs blocked the memory-enhancing effects of caffeine without affecting its anxiogenic effects, whereas knocking out ventral hippocampal A<sub>2A</sub>Rs did not affect the memory-enhancing effects of caffeine but blocked its anxiogenic effects. In addition, there were baseline differences in the behaviors of site-specific A<sub>2A</sub>R knockout mice, and statistical analysis confirmed caffeine-A<sub>2A</sub>R interactions. These results indicate that caffeine modulates memory by inhibiting dorsal hippocampal A<sub>2A</sub>R and modulates anxiety by acting through ventral hippocampal A<sub>2A</sub>R.

To confirm the memory-enhancing effects of dHPC A<sub>2A</sub>R knockout, we assessed the level of SNAP-25. Our study showed that inactivation of dHPC A<sub>2A</sub>R upregulated the density of synaptic proteins, consistent with a previous study that showed that activation of hippocampal A<sub>2A</sub>R is sufficient to attenuate synaptic plasticity and further impair memory (Li et al., 2015). In recent years, synaptic density and synaptic connections in the hippocampus have been associated with learning and memory (Benito and Barco, 2010; Latina et al., 2017), and the overexpression of adenosine receptors revealed a hippocampal LTD-to-LTP shift to impair synaptic plasticity (Temido-Ferreira et al., 2018). Thus, chronic caffeine consumption may affect synaptic function to enhance memory by inhibiting dHPC A<sub>2A</sub>R. However, the specific mechanism remains to be further confirmed.

One of the most important hypotheses of anxiety disorder is inhibition/excitation imbalance (Colic et al., 2018). As vGluT1 is





a glutamatergic-selective marker and labels excitatory glutamatergic neurons (Zheng et al., 2015; Martineau et al., 2017), upregulation of vGluT1 reflects excessive excitation and inhibition/excitation imbalance in vHPC to some extent. It is also consistent with the greater anxiety-like behavior induced by caffeine and dHPC adenosine A<sub>2A</sub> receptor knockout. It is unclear whether caffeine regulates anxiety through the alteration of vGluT1, but the activation of adenosine A<sub>2A</sub> receptor has been reported to reduce GluT and glutamate uptake in cultured astrocytes and gliosomes (Matos et al., 2012). Therefore, the upregulation of vGluT1 may underlie the effect of caffeine on anxiety via inhibition of vHPC adenosine A<sub>2A</sub> receptor.

As a supplement to the brain region-specific knockout of adenosine A<sub>2A</sub> receptor, an optoA<sub>2A</sub>R approach to mimic endogenous A<sub>2A</sub>R signaling was used. Due to the specific construct, overexpression of optoA<sub>2A</sub>R did not generate

baseline effects and caffeine could not react with optoA<sub>2A</sub>R too. After 3-weeks antagonism by caffeine, endogenous A<sub>2A</sub>R signaling was inhibited, optogenetic activation of optoA<sub>2A</sub>R captured the physiological function of the native A<sub>2A</sub>R. Light activation of dHPC adenosine A<sub>2A</sub> receptors reversed the behavioral alterations caused by caffeine, leading to impaired memory, while light activation of vHPC adenosine A<sub>2A</sub> receptors decreased caffeine-induced anxiety, further confirming the dissociation between the roles of the ventral and dorsal hippocampal A<sub>2A</sub> receptors in caffeine's effects. Considering that we examined the behavioral effects of withdrawal from a chronic regime of caffeine administration, caffeine may induce compensatory effects in response to long-term drug exposure. The instant but significant effect of optoA<sub>2A</sub>R suggests the specificity of A<sub>2A</sub>R signaling rather than the compensatory effects.

Since adeno-associated viral vectors driven by either the synapsin- (syn-) or CaMKIIa promoter were employed, our results suggested that the effects of caffeine may result from specific inhibition of neuronal adenosine A<sub>2A</sub> receptors, consistent with previous studies on the role of neuronal adenosine A<sub>2A</sub> receptor in cognition (Kaster et al., 2015; Viana

da Silva et al., 2016; Temido-Ferreira et al., 2018). The prominent cortical connectivity of the dorsal hippocampus and the projection to the anterior cingulate cortices are involved in memory processing (Jones and Wilson, 2005; Lavenex et al., 2006). The CA1 and subiculum of the ventral hippocampus share massive bidirectional connectivity with amygdala nuclei, which plays a key role in control (Pitkanen et al., 2000; Liu and Carter, 2018). The neuronal adenosine A<sub>2A</sub> receptors in those projections may underlie the distinct regulation of adenosine A<sub>2A</sub> receptors in the dHPC and vHPC. No method was designed in this research to confirm the mechanism, and further research is needed to clarify the mechanisms of neuronal A<sub>2A</sub>R-mediated regulation and modulation of caffeine.

## CONCLUSION

In the present study, we blocked and reversed the effects of caffeine by specifically inactivating and activating dHPC/vHPC adenosine A<sub>2A</sub> receptors. For the first time, caffeine was demonstrated to affect memory by inhibiting dorsal hippocampal adenosine A<sub>2A</sub> receptors and affect anxiety by inhibiting ventral hippocampal adenosine A<sub>2A</sub> receptors, explaining how caffeine triggers anxiety while enhancing memory. Our results may help to understand the mechanisms of anxiety and memory and provide an experimental basis for making better use of caffeine while avoiding its side effects.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

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## ETHICS STATEMENT

The animal study was reviewed and approved by The experimental procedures were provided by the Welfare Committee of the Army Medical University.

## AUTHOR CONTRIBUTIONS

YX performed experiments, and wrote the manuscript. PL and YZ designed the study and wrote the manuscript. YN, YZ, YP, and FL performed experiments. YX, YN, YZ, and PL analyzed data. PL and YZ reviewed the manuscript. PL and YZ designed the study and worked on the final approval of the manuscript and financial support.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.807330/full#supplementary-material>

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